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14. ABSTRACT

Almost 50% of all ER+ breast tumors will not respond to endocrine therapy. Resistance to endocrine therapy remains a significant clinical problem and advanced ER+ breast cancer is largely an incurable disease. Endocrine manipulation in sensitive cells can result in the induction of cell death through autophagy and/or apoptosis. We have recently obtained data implicating the unfolded protein response (UPR) as induced by the splicing of X-box binding protein-1 (XBP1) in the regulation of endocrine responsiveness in breast cancer cells. UPR is a key component of the endoplasmic reticulum stress response and has not previously been implicated in endocrine responsiveness. We hypothesize that XBP1(S) is a key regulator of breast cancer cell fate, acting through its regulation of UPR, BCL2, and BCL2:BECN1 heterodimers, and their subsequent effects on autophagy and apoptosis. We will determine how XBP1(S) affects cell fate, evaluating the role of an induction of UPR that activates a prosurvival autophagy. In endocrine sensitive cells, autophagy should persist and become a cell death mechanism that can also initiate apoptosis. In resistant cells, basal autophagy should represent a survival mechanism to deal with the loss of autocrine and other growth factor signaling that accompanies endocrine therapy. We will explore the mechanistic role of XBP1(S) and its integrated signaling through UPR and BCL2 to regulate cell fate in both endocrine sensitive and resistant cells.

15. SUBJECT TERMS

Antiestrogens, aromatase inhibitors, BCL2 family, bioinformatics, drug resistance, endoplasmic reticulum, Faslodex, Fulvestrant, gene networks, mitochondria, molecular signaling, Tamoxifen, unfolded protein response.

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INTRODUCTION

In 2008, over 40,000 American women will die of breast cancer [1]. In the same period, there will be over 178,000 newly diagnosed cases of invasive breast cancer, almost 70% of which will be estrogen receptor-α positive (ER+) [2,3]. However, 50% of all ER+ breast tumors will not respond to endocrine therapy [4]. Tamoxifen produces an overall 26% proportional reduction in mortality [5] but many ER+ tumors that show an initial response to tamoxifen eventually recur [4]. Resistance to endocrine therapy remains a significant clinical problem and advanced ER+ breast cancer is largely an incurable disease. Endocrine manipulation in sensitive cells can result in the induction of cell death through autophagy and/or apoptosis. However, the control of these processes, and an understanding of how the dual nature of autophagy is regulated in breast cancer cells (autophagy can be prodeath or prosurvival), is largely unknown. We have recently obtained data implicating the unfolded protein response (UPR) as induced by the splicing of X-box binding protein-1 (XBP1) in the regulation of endocrine responsiveness in breast cancer cells. UPR is a key component of the endoplasmic reticulum stress response and has not previously been implicated in endocrine responsiveness.

We propose that XBP1 uses a specific cellular stress response mechanism (the unfolded protein response), members of the BCL2 gene family, and two other genes, *i.e.,* beclin 1 (BECN1) and MYC to mediate this control of cell fate. The choice to live or die is a critical decision for a breast cancer cell, and a greater understanding of how this choice is regulated is needed. This IDEA award would allow us to explore, in a timely and effective manner, these very recent observations that have lead directly to the construction of our novel hypothesis.

The proposed research could lead to better approaches to predict an individual patient's responsiveness to endocrine therapies and to the development of new strategies to improve the efficacy of endocrine therapies and increase overall survival. For example, measuring the coexpression of activated XBP1 and its key downstream targets that regulate cell survival could be used to more accurately predict the sensitivity of a tumor to endocrine therapy. Inhibiting the activation of XBP1 could either prevent the development of resistance or restore sensitivity.

BODY

Overview: XBP1 is a key regulator of cell fate, acting through its regulation of UPR, BCL2/BECN1 and their subsequent effects on autophagy and apoptosis. Specifically, we hypothesized that XBP1(S) uses UPR (proautophagy) and BCL2 (antiapoptosis) and BCL2:BECN1 interactions (antiautophagy) to regulate the balance between autophagy and apoptosis and to determine breast cancer cell fate in response to antiestrogens. In this final year of the grant (no cost extension), we were able to determine the complex signaling mechanism by which c-MYC and BCL2/BECN1 control cell survival in antiestrogen resistant cells. We were able to initiate but have not yet completed the animal studies outlined in Specific Aim1, mostly due to some technical difficulties with XBP1(S) siRNA and stable XBP1(S) overexpression have also initially delayed some of the gene network modeling. However, we are committed to completing and publishing these exciting studies once they are completed.

Hypothesis: We hypothesize that XBP1(S) is a key regulator of breast cancer cell fate, acting through its regulation of UPR, BCL2, and BCL2:BECN1 heterodimers, and their subsequent effects on autophagy and apoptosis. Specifically, we hypothesize that XBP1(S) uses UPR (proautophagy) and BCL2 (antiapoptosis) and BCL2:BECN1 interactions (antiautophagy) to regulate the balance between autophagy and apoptosis and to determine breast cancer cell fate in response to antiestrogens and aromatase inhibitors (which we will model with estrogen deprivation).

Specific Aims: We will use a series of human breast cancer cell lines/variants and apply established and state-of-the art methods to address our specific aims. We will explore the mechanistic role of XBP1(S) and its integrated signaling through UPR and BCL2 to regulate cell fate in both endocrine sensitive and resistant cells.

AIM 1: We will determine how XBP1(S) affects cell fate, evaluating the role of an induction of UPR that activates a prosurvival autophagy. In endocrine sensitive cells, autophagy should persist and become a cell death mechanism that can also initiate apoptosis. In resistant cells, basal autophagy should represent a survival

mechanism to deal with the loss of autocrine and other growth factor signaling that accompanies endocrine therapy, with the switch to prodeath signaling being concurrently suppressed.

AIM 2: We will determine how XBP1(S) signals (*e.g.,* through BCL2 and BECN1) to affect endocrine responsiveness and cell survival. We will then use these data to build an interactive *in silico* model of how this signaling operates (how the nodes are connected and function) in the context of endocrine responsiveness.

KEY RESEARCH ACCOMPLISHMENTS

2009

- Endocrine resistance in breast cancer cells over-expressing XBP1 is linked to increase in autophagy and UPR
- Knockdown of XBP1 in resistant cells down-regulates antiapoptotic BCL2
- Resistant cells are more sensitive to growth inhibition by small molecule BCL2 and MYC inhibitors
- Published two peer-reviewed articles (see Reportable Outcomes)

2010

- We determined the central role for autophagy in antiestrogen resistance and the important roles played by BCL2 and potentially also a role for the up-regulation of MYC in these resistant cells.
- We showed that combination therapy that includes an antiestrogen and a BCL2 inhibitor can significantly re-sensitize antiestrogen resistant breast cancer cells.
- Published one peer-reviewed article and two meeting abstracts (see Reportable Outcomes below).

2011

- From the tasks carried out in Year 3 of this grant, we were able to highlight the essential role of c-MYC in antiestrogen resistance. Inhibition of c-MYC with 10058-F4 (small molecule inhibitor for c-MYC/MAX heterodimer) re-sensitized antiestrogen resistant cells to Faslodex. Combination therapy that includes an antiestrogen, a BCL2 inhibitor and a c-MYC inhibitor can significantly re-sensitize antiestrogen resistant breast cancer cells. However, the level of inhibition with this combination was comparable to the c-MYC plus Faslodex alone indicating that c-MYC may be involved in regulation of BCL2 in antiestrogen resistance.
- Based on our findings, we published two peer-reviewed academic reviews that include the work funded through this award and two meeting abstracts (see Reportable Outcomes).

2012

- c-Myc is upregulated in endocrine resistant breast cancer and inhibition of Myc-Max heterodimer induces apoptosis without affecting cell cycle profile in antiestrogen resistant LCC9 cells
- Successful identification of small molecule inhibitor to IRE1a (NPPTA) induces cell death (apoptosis and autophagy) in breast cancer cells but not in normal mammary gland epithelial cells. NPPTA synergized with antiestrogens (Tamoxifen and ICI 182,780) in both LCC1 (estrogen-independent and

antiestrogen sensitive) and LCC9 (estrogen-independent and antiestrogen resistant)

- Antiestrogen resistant breast cancer cells use a constant basal level of autophagy to promote cell survival.
- XBP1(S) overexpression enhances tumor growth in an orthotopic breast cancer model.

REPORTABLE OUTCOMES

Manuscripts:

1. Clarke et al. Cancer Res., 72:1321-31, 2012.
2. Clarke *et al.* Horm Mol Biol Clin Invest, 5: 35-44, 2011.
3. Cook *et al.* Expert Rev Anticancer Aug;11(8):1283-94. 2011.
4. Crawford et al., PLoS One. 5:e8604, 2010.
5. Shajahan, et al., Drug News Perspect 22: 241-246, 2009.
6. Clarke, et al., J Steroid Biochem Mol Biol, 114: 8-20, 2009.
7. Shajahan et al., FASEB J: *Inhibition of c-MYC re-sensitizes endocrine resistant breast cancer cells by down-regulating BCL2 and increasing apoptosis, in preparation.*
8. Shajahan et al., *Nat Chem Biol: Small molecule inhibition of IRE1a prevents XBP1 splicing and selectively induces cell death in antiestrogen resistant breast cancer cells, in preparation.*

Patent:

1. International Application No. PCT/US12/032110. *"Small Molecule Inhibitors of XBP1 Splicing"* (pending: application filed)

Abstracts:

- AACR 2010 abstract #2919: XBP1 and the unfolded protein response in antiestrogen resistance in breast cancer. Ayesha N. Shajahan, Rebecca B. Riggins, Alan Zwart, F. Edward Hickman, Robert Clarke.
- AACR 2010 abstract #4601: XBP1 regulated function of c-MYC and BCL2 in antiestrogen resistance in breast cancer. Lauren M. McDaniel, Ayesha N. Shajahan, Robert Clarke.
- AACR 2011: XBP-1 promotes cell survival by activating the unfolded protein response (UPR) in antiestrogen resistant breast cancer cells. Ayesha N. Shajahan, Katherine Cook, F. Edward Hickman and Robert Clarke.
- Experimental Biology 2011: The unfolded protein response (UPR) in antiestrogen resistance in breast cancer. Ayesha N. Shajahan, F. Edward Hickman, Katherine Cook, Alan Zwart and Robert Clarke.
- AACR 2012: Measurement of autophagy in sensitive versus resistant breast cancer cells. Ayesha N. Shajahan[1], Rory Olson[2], Mathew Webb[2], Jennifer Samoy[2], John Zielinski[2], Michael Mullenix[2], Robert Clarke[1]. [1]Georgetown University, Lombardi Comprehensive Cancer Center, Washington, DC; [2]Enzo Life Sciences, Ann Arbor, MI.

CONCLUSION

Our data from this BCRP award strongly support a central role for autophagy and UPR, incorporating the activities of BCL2, MYC, and XBP1. The results suggest that resistant cells are more reliant upon BCL2 and MYC for cell survival. Use of small molecule inhibitors of BCL2 or MYC may be candidates for consideration as new drug therapies is exciting – several new agents against these targets are now entering clinical trials in other cancers. We hope to be able to eventually use the data from this grant to design clinical trials to directly

test this hypothesis and potentially improve endocrine therapies for women with breast cancer.

We have applied for a patent for small molecule inhibitors for IRE1a (analogs of NPPTA) that exhibit promise as potent inhibitors of antiestrogen resistant breast cancer cells. Our studies also indicate that NPPTA inhibits breast cancer cells but not normal mammary epithelial cells. This finding is consistent with our overall hypothesis that endocrine resistant breast cancer cells have increased levels of UPR-regulated autophagy to promote cell survival. These data will be used to obtain funding to support further preclinical *in vivo* validation of NPPTA analogs and further investigation of this novel group of compounds to overcome endocrine resistance in breast cancer.

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SUPPORTING DATA: figures/legends

Fig 1: Cleavage of the autophagy-associated marker LC3II is enhanced in T47D/XBP1 cells. A, Western blot. *denotes LC3II cleavage product. B, quantitation of LC3II:actin ratio. C=empty vector; TUN = 2μg/ml (positive control), FAS=100 nM.

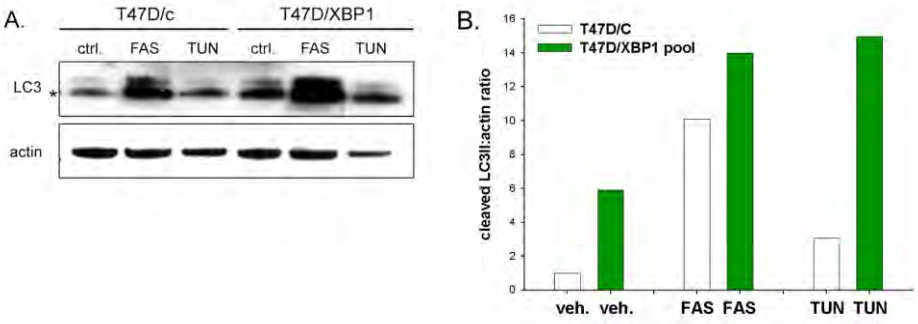
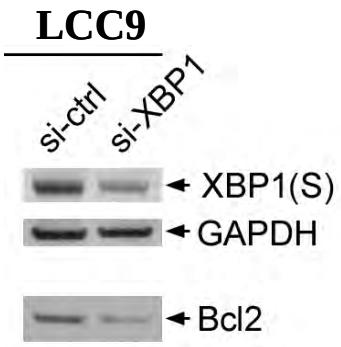
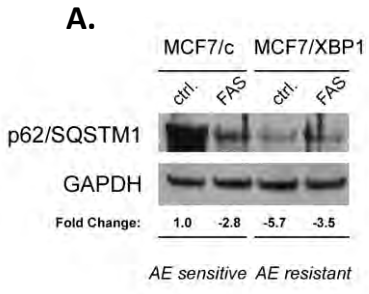


Fig 2: Knockdown of XBP1 by siRNA reduces BCL2 expression. XBP(s) was knocked down in LCC9 cells using siRNA or the relative control siRNA. Total protein levels were determined by Western blot analyses using specific antibodies.





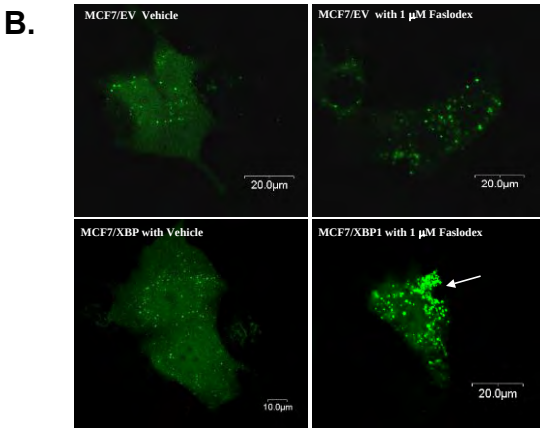


Fig 3: Autophagy is enhanced upon FAS treatment and/or XBP1 over-expression in ER+ breast cancer cell lines. A, MCF7 stably expressing XBP1 cDNA or the empty vector control (c) were treated with FAS or ethanol control (ctrl.) vehicle prior to lysis and immunoblotting using standard procedures. Fold change values indicate the ratio of each target protein to the loading control, and are normalized to empty vector, vehicle-treated cells. B, Expression of GFP-LC3II in MCF7/EV or MCF7/XBP1 stable cells. MCF7/XBP1cells treated with 1uM FAS showed increased level of GFP-LC3II.

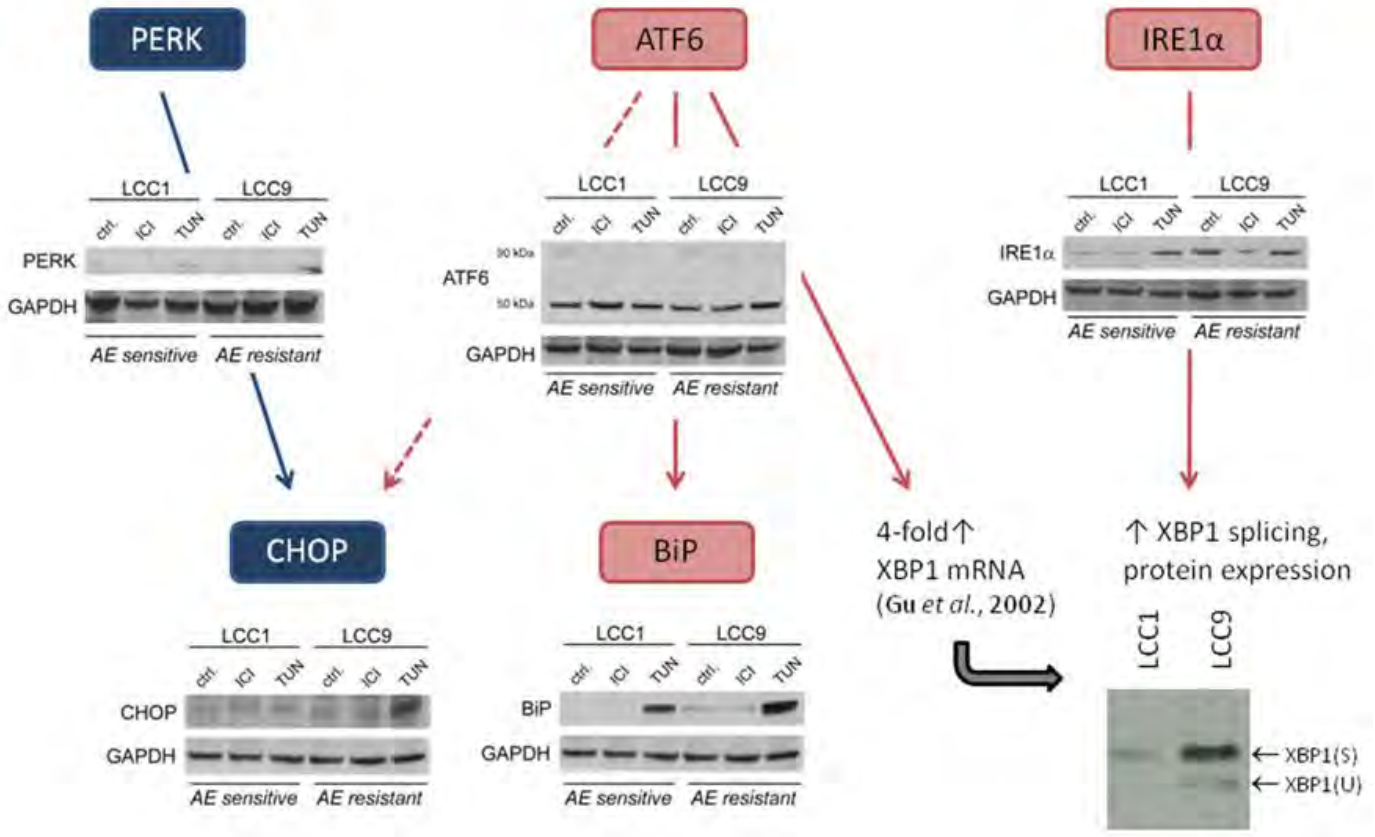


Fig 4: UPR signaling is enhanced in resistant LCC9 cells, particularly via the IRE1α arm. Total proteins were extracted from LCC1 or LCC9 cells that were treated with either vehicle alone, 100 nM ICI 182,780 or 2 μg/ml tunicamycin and analyzed by Western blotting.

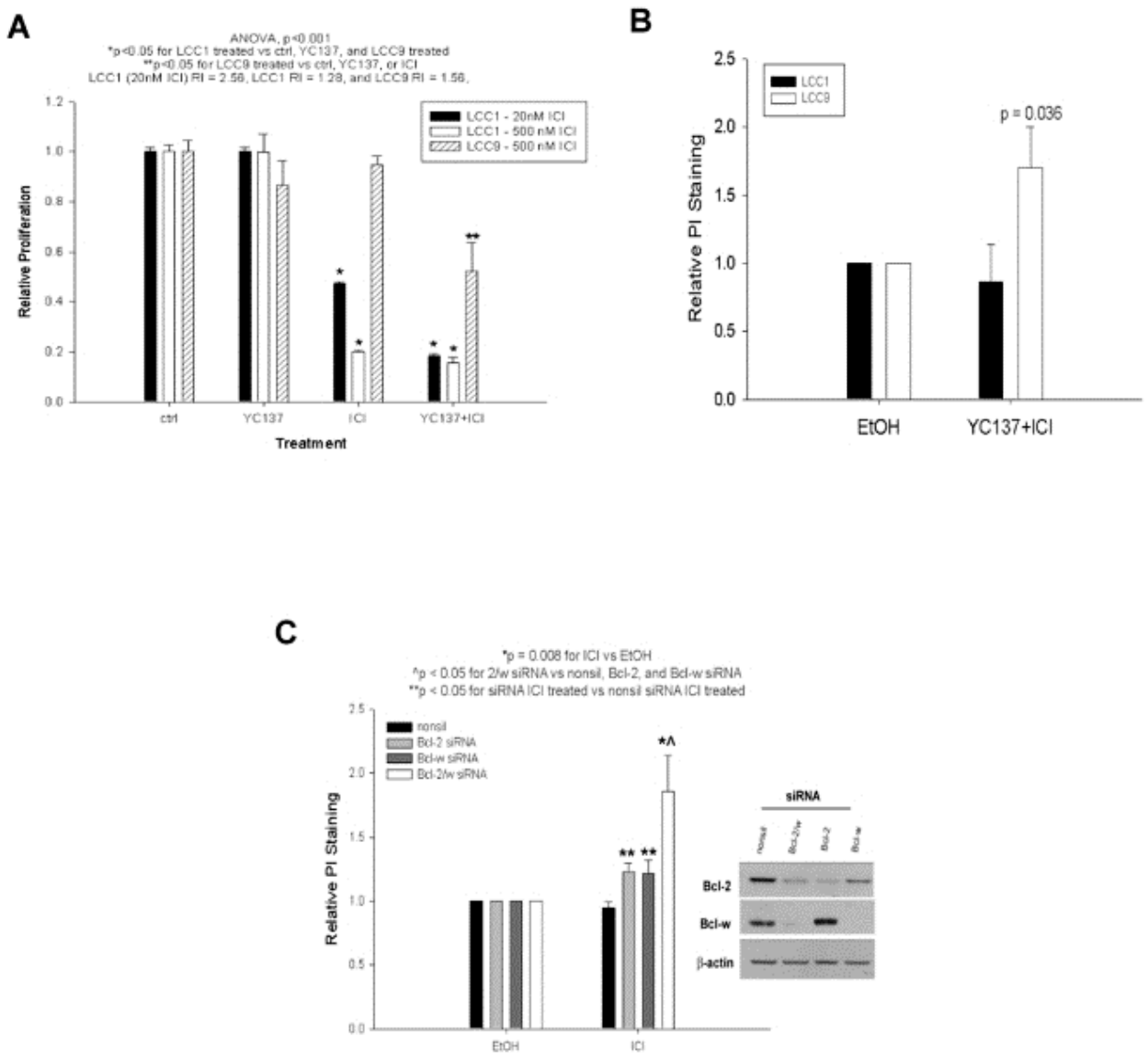


Figure 5: BCL-W and BCL2 inhibition increases sensitivity to ICI 182,780 and increases necrosis in MCF-7/LCC9 cells. (**A**) Cells were treated with YC137 and/or ICI for 7-days. Bars represent the mean ± SE of relative cell proliferation (normalized to EtOH treated controls) for a single representative experiment performed in triplicate. (**B**) Cells were treated and stained with propidium iodide (PI). Bars represent the mean ± SE of relative PI staining (normalized to control EtOH treated cells) for three independent experiments. (**C**) Cells were transfected with siRNA and stained with PI. Inset, a representative blot showing BCL-W and BCL2 siRNA knockdown (Crawford *et al.,* PLoS One. 5:e8604, 2010 [listed in "REPORTABLE OUTCOMES"]).

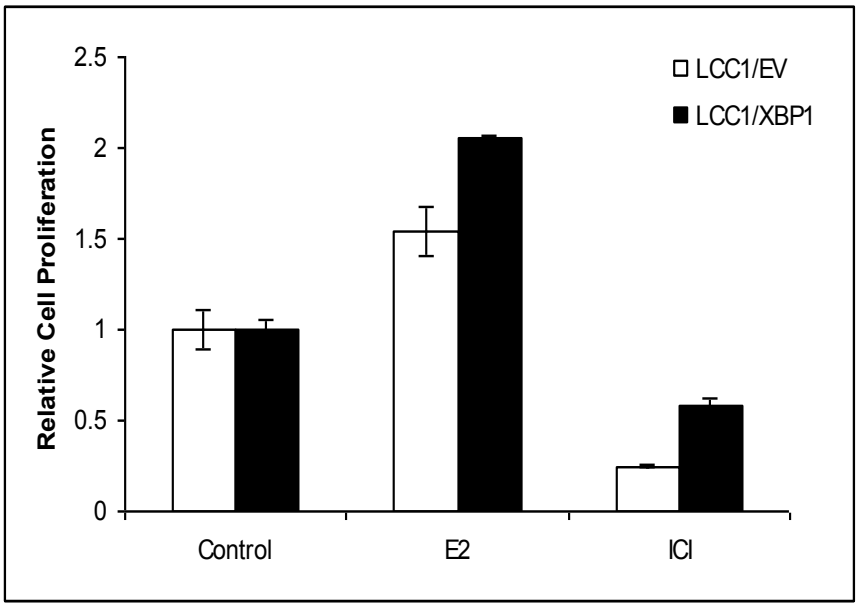


Figure 6: Transient expression of XBP1 in LCC1 (antiestrogen sensitive) breast cancer cells increased response to estradiol (E2, 10 nM) but decreased response to Faslodex/ICI 182, 780 (100 nM) compared with that in cells transfected with empty vector (EV). Cell proliferation was measured after treatment for 6 days. To measure cell viability, cells were plated in 96-well plastic tissue culture plates at a density of $5x10^3$ cells/well. After treatment, cell culture media was removed and plates were stained with 100 ml/well of a solution containing 0.5% crystal violet and 25% methanol, rinsed with deionized water, dried overnight, and resuspended in 100 ml citrate buffer (0.1M sodium citrate in 50% ethanol) to assess cell density. Intensity of crystal violet staining, assessed at 570 nm and quantified using a Vmax Kinetic Microplate Readerand Softmax software (Molecular Devices Corp., Menlo Park, CA).

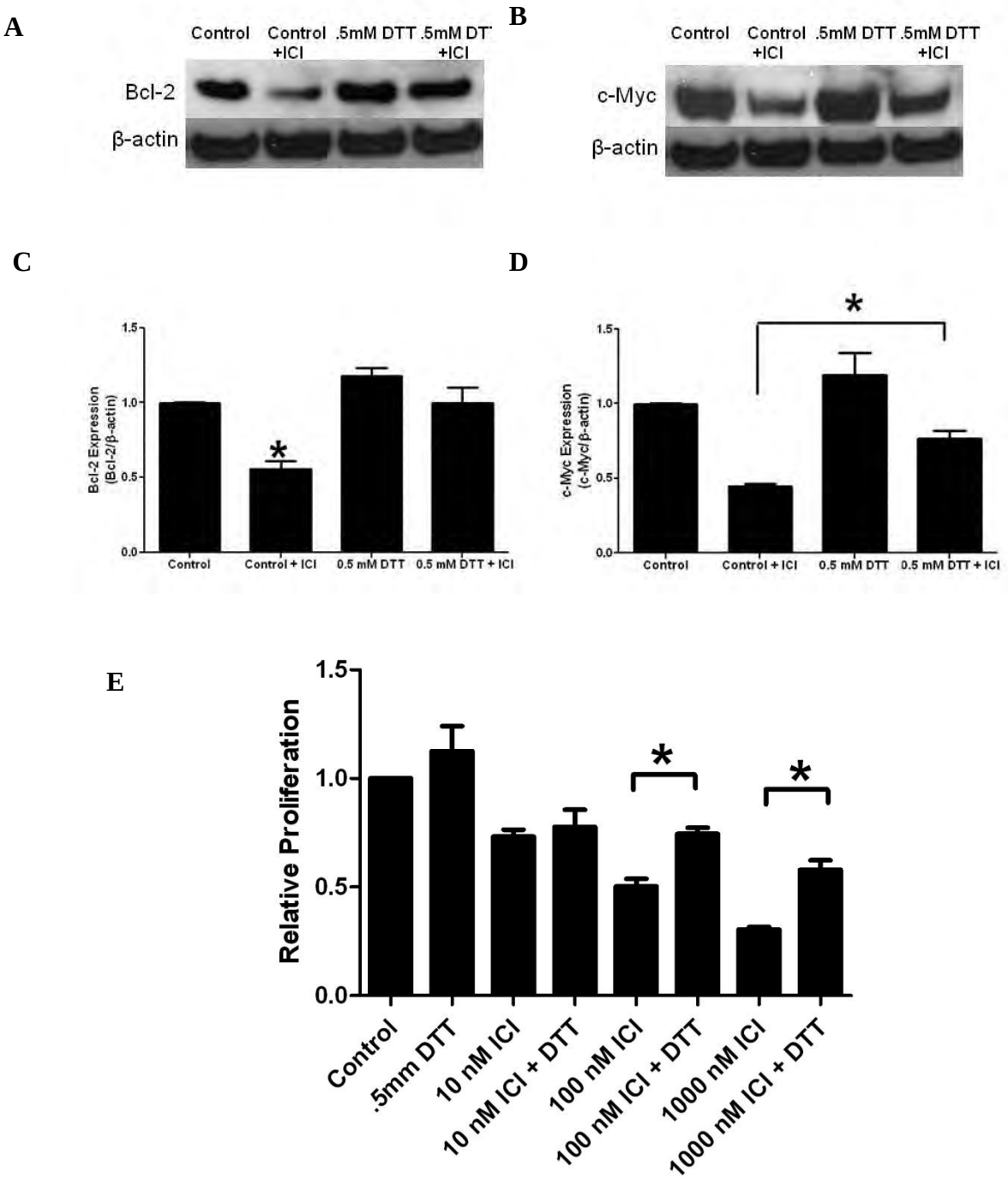


Figure 7: Induction of unfolded protein response by low-dose DTT (0.5 mM) increases resistance to antiestrogens in sensitive LCC1 breast cancer cells. *A-D*, Upregulation of autophagy using low levels of DTT (0.5 mM; DTT commonly used to induce unfolded protein response) in antiestrogen sensitive LCC1 cells, increases BCL2 and c-MYC levels. *E*, Low dose DTT-mediated upregulation of autophagy protect LCC1 cells from Faslodex-induced apoptosis. *, $p<0.05$ by a Student's t-test.

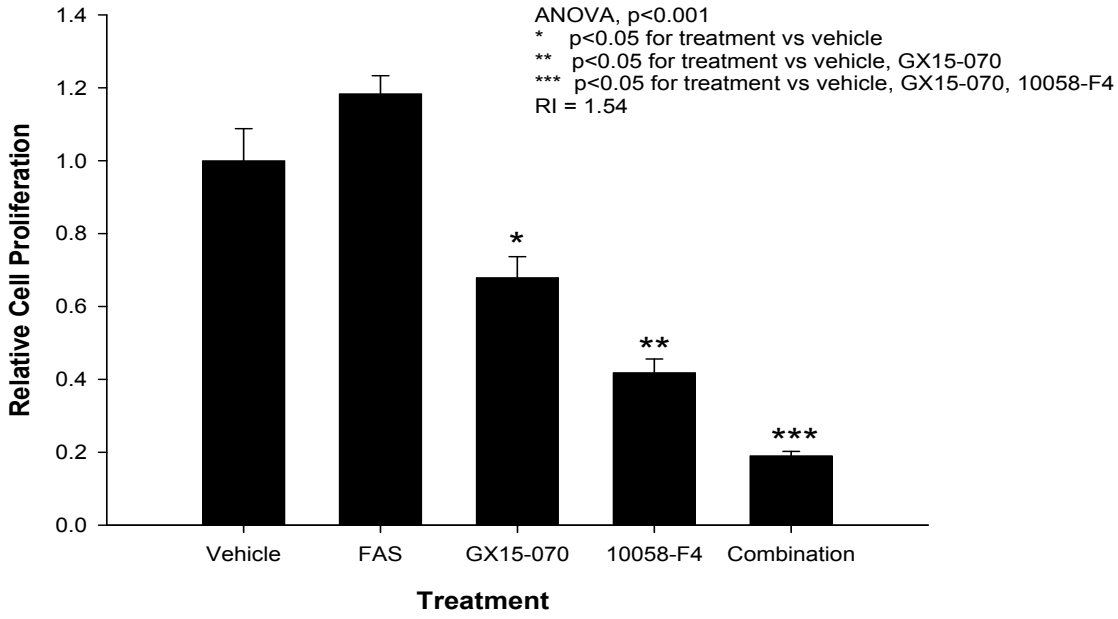
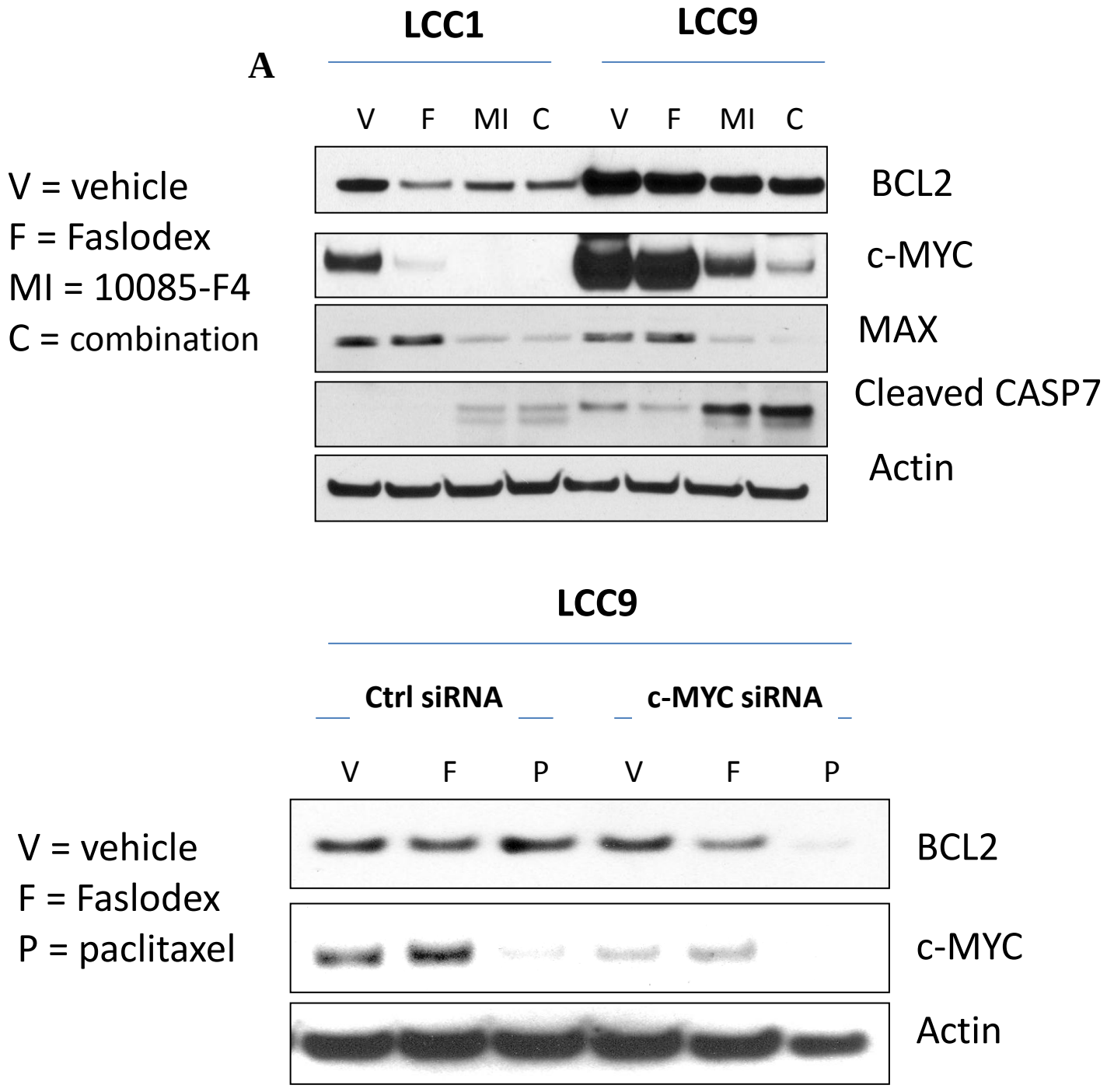


Figure 8: Combination of Faslodex (ICI 182,780; 100 nM), 10058-F4 (MYC inhibitor, 25 μM) and GX15-070 (BCL2 inhibitor, 100 nM) synergistically inhibited cell proliferation within 72 h in antiestrogen-resistant LCC9 cells.

Figure 9: *A*, 10058-F4 (25 nM) alone or in combination with ICI 182,780 (100 nM) decreases MYC, MAX and BCL2 protein levels. *B*, MYC siRNA reduced BCL2 protein levels in combination with ICI 182,780 or paclitaxel (10 nM, positive control for apoptosis).



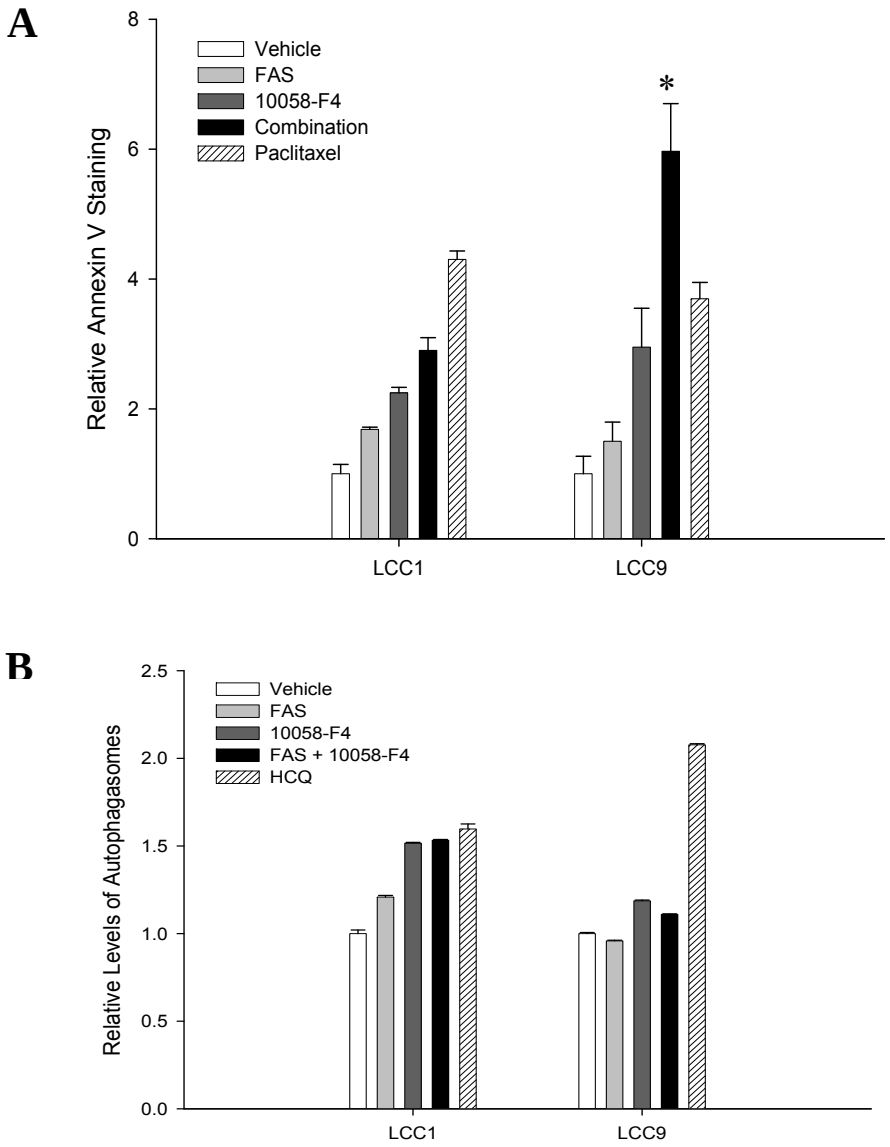


Figure 10: *A*, Combination of 10058-F4 (25 μM) and 100 nM ICI induced significantly higher levels of apoptosis in LCC9 cells at 48 h compared to LCC1 cells. Paclitaxel (10 nM) was used as a positive control for apoptosis; *, $p<0.05$, ANOVA. *B*, Levels of autophagosome formation as detected by a derivative of monodansylcadaverine (Cyto-ID; Enzo Life Sciences, Farmingdale, NY) only showed a modest increase in autophagosomes LCC9 cells following treatment with 10058-F4 or combination with ICI at 48 hr. HQC, hydoxychloroquine was used as a positive control for induction of autophagy.

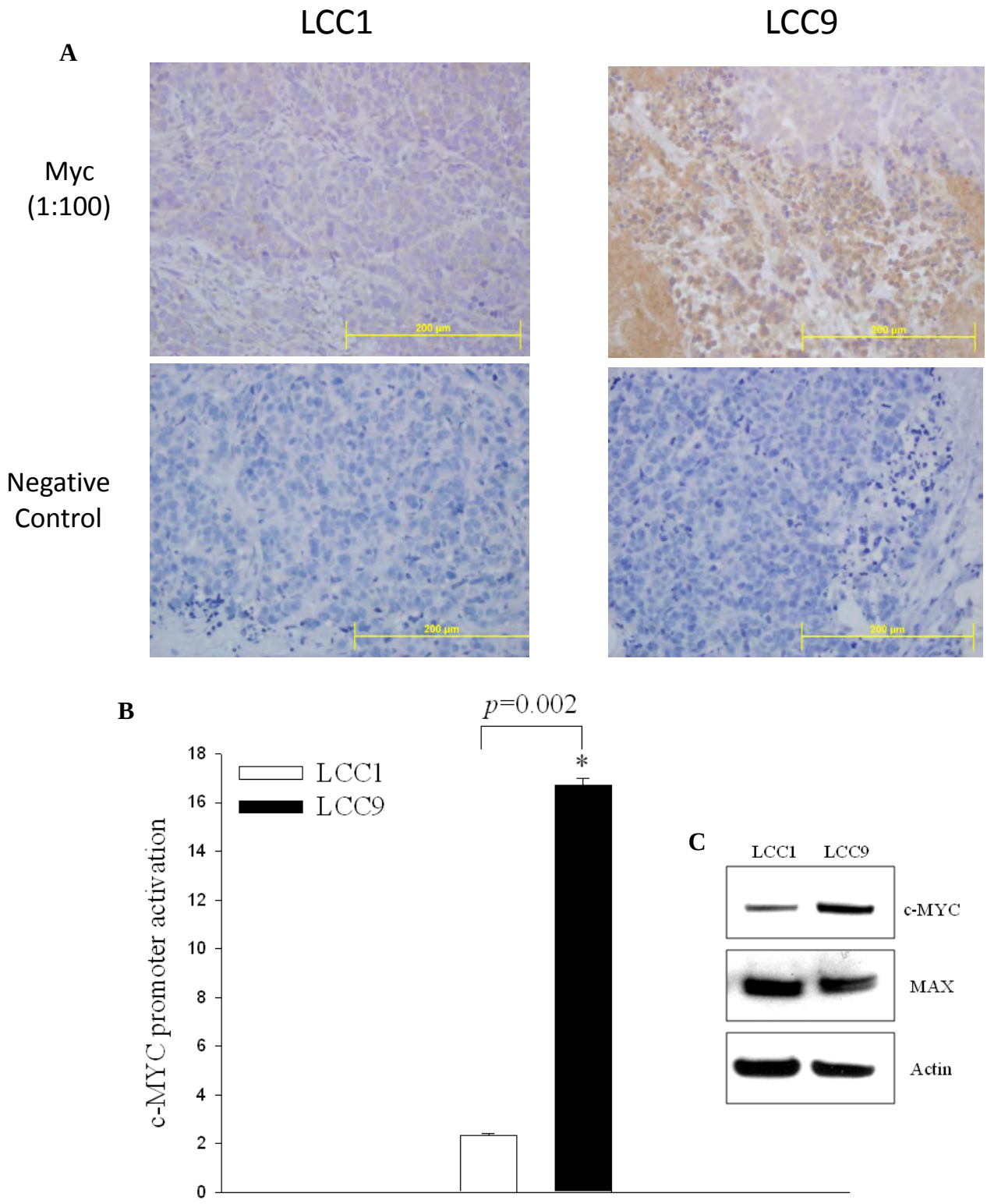


Figure 11: MYC expression is increased in antiestrogen resistant LCC9 cells and xenografts. *A*, Immunohistochemistry (IHC) analysis of untreated orthotopic LCC1 and LCC9 xenografts showed increased expression of MYC protein in LCC9 xenografts. *B*, MYC-promoter activation in significantly increased in LCC9 cells. *C*, Western blot analyses of total protein show increased levels of MYC in LCC9 compared to LCC1 cells but the levels of MAX remain unchanged.

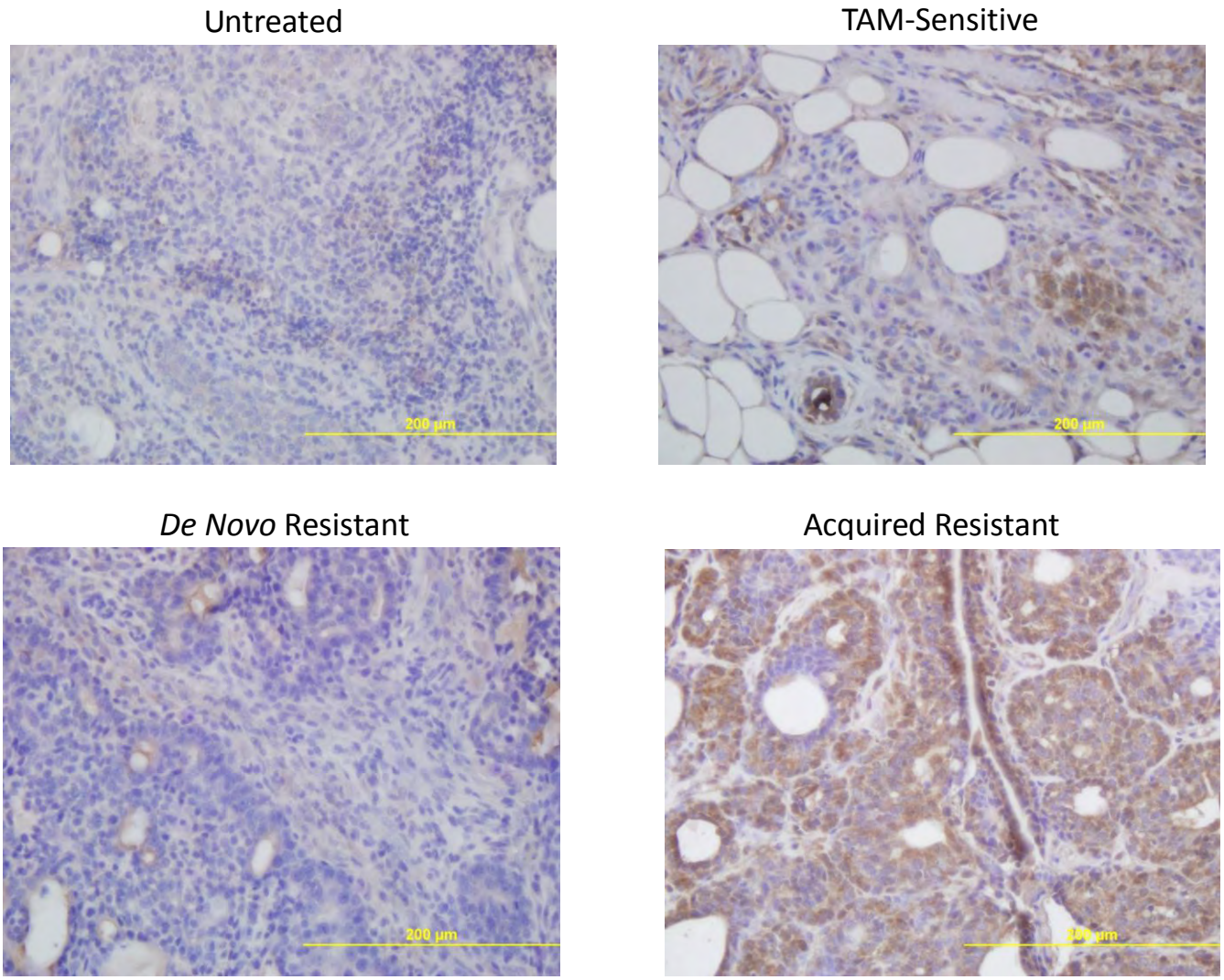


Figure 12: In a 7,12-dimethylbenz[*a*]anthracene *(*DMBA)-induced rat tumor model, c-Myc protein levels were higher in acquired resistant mammary tumors when compared with untreated, complete response, and de novo resistant tumors in response to TAM. These data strongly suggest that increase in MYC protein expression correlates with increased antiestrogen resistance in breast cancer cells *in vitro* and *in vivo*.

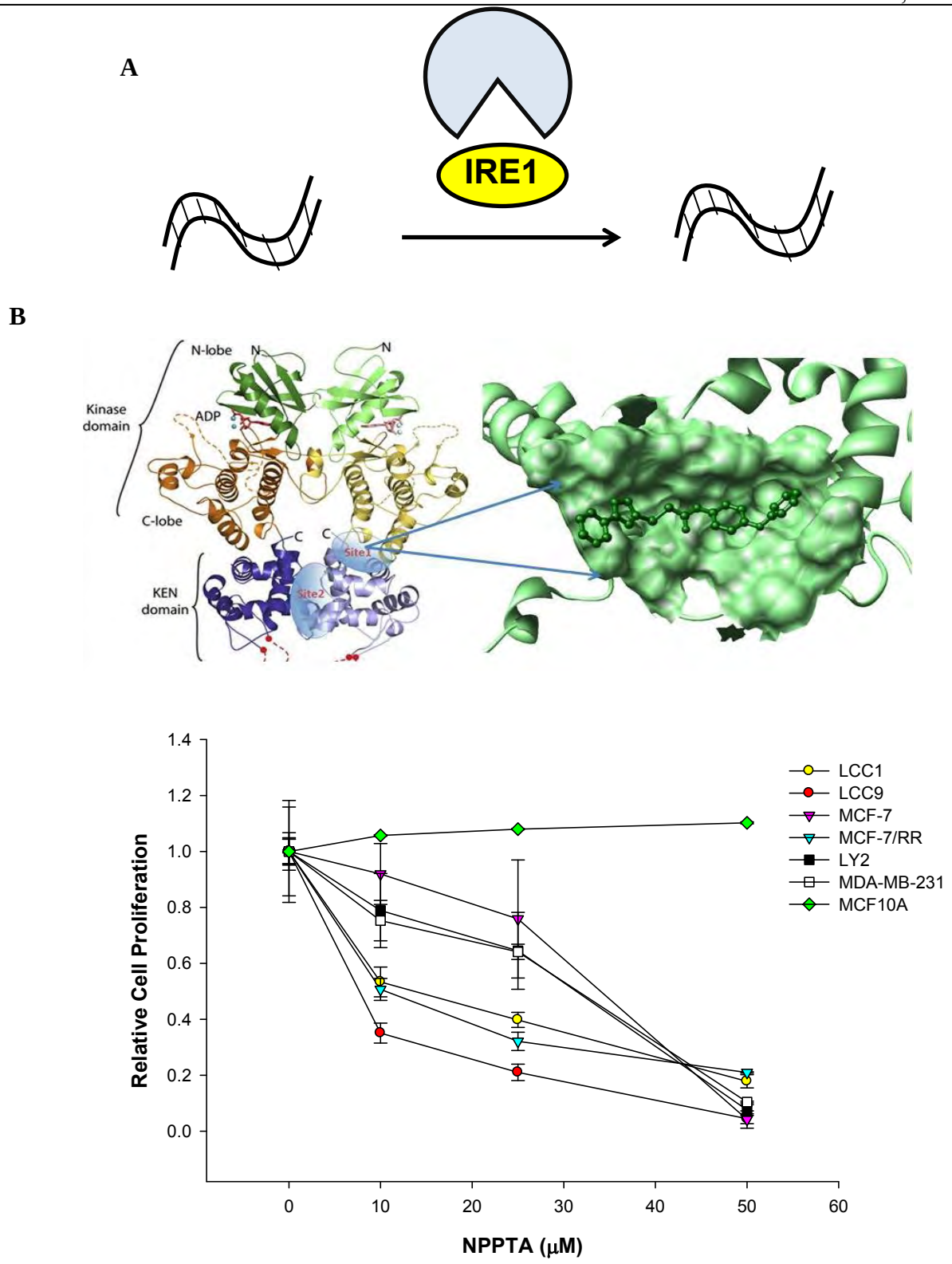


Figure 13: *A* and *B*, N-(4-Phenoxy-phenyl)-2-(5-pyridin-3-yl-2H-[1,2,4]triazol-3-ylsulfanyl)-acetamide (NPPTA) inhibits the endoribonuclease activity (but not kinase) of IRE1. NPPTA interacts with the protein-protein interface between the nuclease (KEN) and kinase domains. *C*, NPPTA inhibits cell proliferation dose-dependently in antiestrogen resistant cells while not having any effect on normal (immortalized) mammary gland cells.

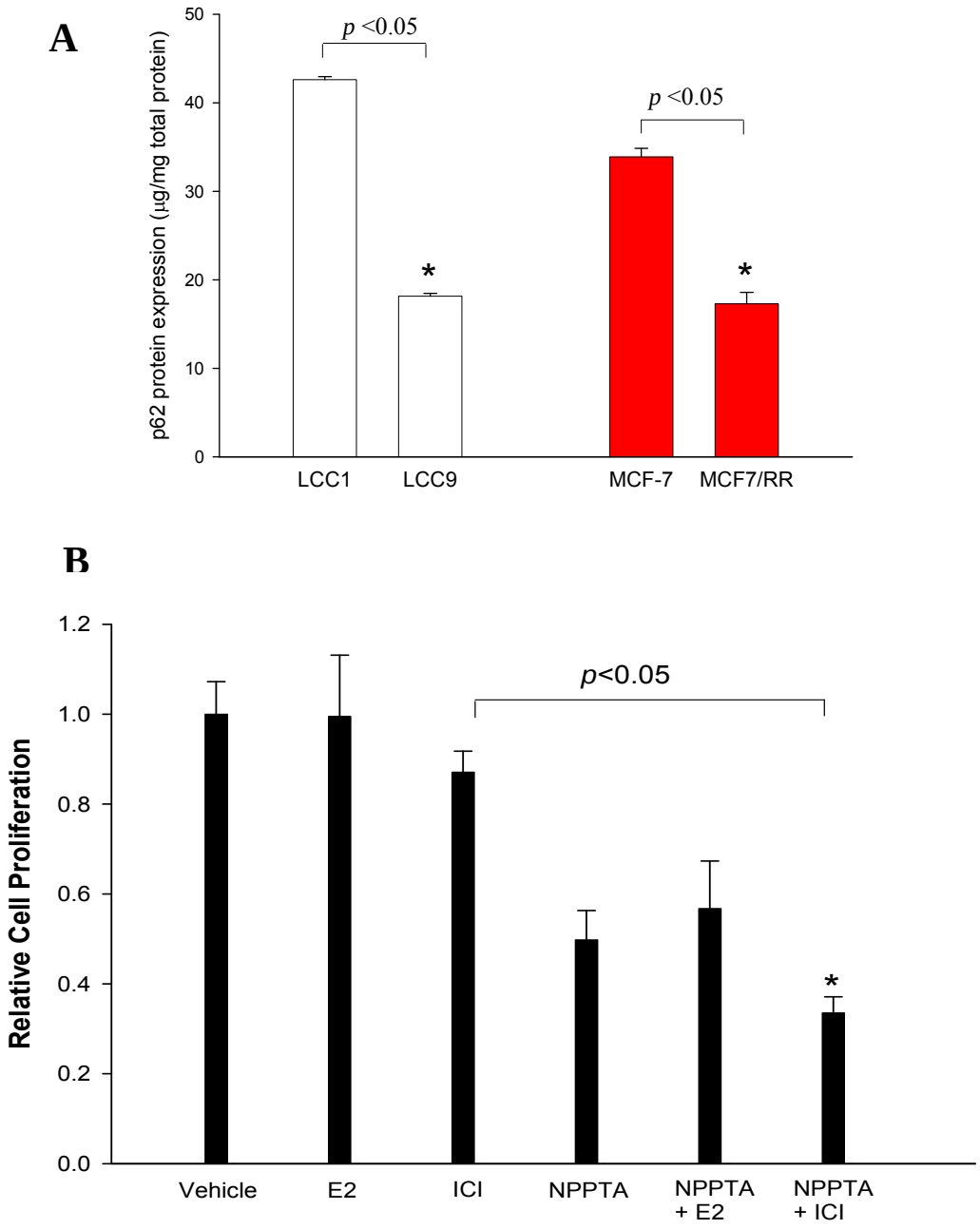


Figure 14: *A*, NPPTA re-sensitize LCC9 cells to ICI. 17β-estradiol (E2) cannot rescue the effects of NPPTA in LCC9 cells. While ICI alone is inactive in LCC9 cells, NPPTA (10 µM) and ICI (500 nM) show a beneficial interaction (modest synergy; RI=1.27) when combined and treated for 72 h, *, p<0.05, ANOVA. *B*, Antiestrogen resistant cells show increased levels of basal autophagy. p62/SQSTM1 (marker for autophagy function) protein levels were measured in LCC1, LCC9, MCF7 (antiestrogen sensitive) and MCF7/RR (Tamoxifen resistant) cells under basal condition. p62 levels were significantly lower (indicates increased levels of autophagy) in LCC9 cells compared to that in LCC1 cells and in MCF7/RR cells compared to that in MCF7 cells p<0.05 by Student's *t* test.

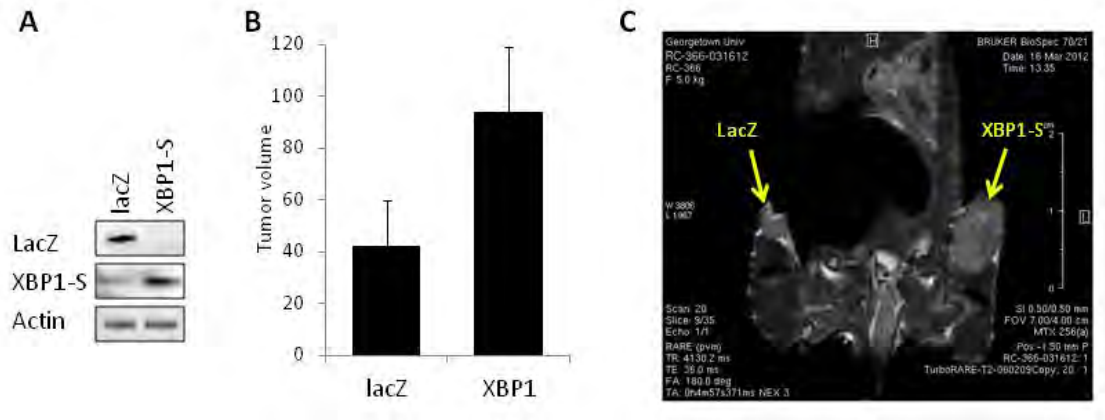


Figure 15: To confirm the *in vitro* results we explored whether XBP1 overexpression can drive tumorigenesis *in vivo* by comparing growth of MCF7-LacZ and MCF7-XBP1(S) (MCF7 cells overexpressing the active form of XBP1; **A**) tumors in an orthotopic nude mouse model. Our results clearly show that XBP1(S) overexpression enhanced tumor growth (**B-C**). *A***,** XBP1(S) and LacZ overexpressing MCF7 cells were generated with lentiviral transfection and selected with puromycin, and XBP1(S) overexpression was confirmed in a Western blot. We also initiated a treatment study with TAM (5mg/pellet/60-day release; Innovative Research of America, FL, USA) on tumor bearing mice to evaluate the effect of XBP1(S) overexpression on treatment response, but the number of treated tumors is still too low for data analysis. The MCF7-LacZ tumors grew much slower than MCF7-XBP1(S) tumors, thus, in a treatment study they need to be grown in different animals (we had inoculated on opposite flanks, which is normally a good design). *B*, One million of XBP1(S) and LacZ cells were injected orthotopically into contralateral abdominal #4 mammary fat pads of ovariectomized nude mice (Harlan, USA) supplemented with an estrogen pellet (0.72 mg, 60-day release; Innovative Research of America, USA) *s.c*., *i.e*. each mouse had both XBP1(S) and LacZ xenografts. Tumor growth was measured weekly and cumulative tumor burden of MCF7-LacZ (control, n=6) and MCF7-XBP1(S) (n=6) tumors after 4 weeks is shown. *C*, In addition, the LacZ and XBP1(S) tumors of one mouse were imaged using a Bruker Biospec 7 T small animal MRI, and, next, will also be analyzed by high resolution localized spectroscopy in order to reveal potential metabolic differences between the two tumor types, in addition to the apparent size difference (arrows).

Endoplasmic reticulum stress, the unfolded protein response, and gene network modeling in antiestrogen resistant breast cancer

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Running title. *Unfolded protein response*

Abstract

Lack of understanding of endocrine resistance remains one of the major challenges for breast cancer researchers, clinicians, and patients. Current reductionist approaches to understanding the molecular signaling driving resistance have offered mostly incremental progress over the past 10 years. As the field of systems biology has begun to mature, the approaches and network modeling tools being developed and applied therein offer a different way to think about how molecular signaling and the regulation of critical cellular functions are integrated. To gain novel insights, we first describe some of the key challenges facing network modeling of endocrine resistance, many of which arise from the properties of the data spaces being studied. We then use activation of the unfolded protein response (UPR) following induction of endoplasmic reticulum stress in breast cancer cells by antiestrogens, to illustrate our approaches to computational modeling. Activation of UPR is a key determinant of cell fate decision making and regulation of autophagy and apoptosis. These initial studies provide insight into a small subnetwork topology obtained using differential dependency network analysis and focused on the UPR gene XBP1. The XBP1 subnetwork topology incorporates BCAR3, BCL2, BIK, NFκB, and other genes as nodes; the connecting edges represent the dependency structures amongst these nodes. As data from ongoing cellular and molecular studies become available, we will build detailed mathematical models of this XBP1-UPR network.

Introduction

Despite over 30 years of relatively safe and effective endocrine therapies, from the advent of Tamoxifen and antiestrogens (AE) to the more recent application of third generation aromatase inhibitors (AI), many estrogen receptor-alpha (ER) positive breast cancers either fail to respond (*de novo* resistance) or eventually recur on or after endocrine therapy (acquired resistance) (1, 2). The major reductions in the risks of recurrence and death that women with an ER+ breast cancer derive from these therapies represent a major achievement. Nonetheless, our lack of understanding of endocrine resistance remains one of the major challenges for breast cancer researchers, clinicians, and patients (3, 4). While resistance to hormonal therapies is an active area of research, and several genes and signal transduction pathways have been implicated in the underlying processes (5-7), our understanding of the fundamental molecular regulatory networks that drive cell survival and proliferation in this phenotype (or phenotypes) is clearly inadequate. Recent advances in the molecular classification of breast cancers (8, 9) have done little to change routine clinical practice for the management of ER+ breast cancers, which represent 70% of all newly diagnosed breast cancer each year. Unfortunately, few effective new strategies to treat advanced, endocrine resistant, ER+ breast cancer have emerged in recent years. Indeed, metastatic breast cancer remains largely an incurable disease.

To create new opportunities for drug discovery and therapeutic interventions, we believe it is essential to acquire first an adequate understanding of the true nature of the molecular interactions responsible for the endocrine resistance phenotype (6). Current approaches to understanding molecular signaling appear limited and have offered somewhat slow and incremental progress over the past 10 years. As the field of systems biology has begun to mature, the approaches and tools being developed therein may provide a different way to think about how molecular signaling and the regulation of critical cellular functions are integrated. One key difference in a systems approach, compared with the more common reductionist approach, is the application of computational and mathematical modeling to represent dynamic system function. These modeling tools are often applied to the high dimensional data sets obtained from microarray, proteomic, and sequencing technologies. However, there are often poorly understood challenges in the analysis of such large data sets that reflect unique properties of high dimensional data spaces (10, 11).

Network modeling and endocrine responsiveness

A primary reductionist focus on individual genes and/or simple signal transduction pathways is likely

one limitation of our ability to derive fundamentally new insights into the molecular underpinning of the phenotype (or perhaps phenotypes) that is resistance (and often crossresistance) to AEs and AIs. These types of signaling-based studies are frequently based on hypotheses framed in the context of the limitations of transduction pathways as understood from largely static models, such as those represented in the KEGG or Biocarta databases, or as constructed *de novo* from modeling tools such as Ingenuity Pathway Analysis or Ariadne Pathway Studio (6). Many of these tools have their uses but they are limited by the frequent inability to account for cellular context and molecular dynamics. Moreover, the true complexity of molecular signaling is probably affected by biological properties, rules, or functions that we do not yet fully understand. For example, the existence and potentially powerful regulatory influences of miRNAs have been only relatively recently discovered. We have long advocated for a more network-based approach (12) but the tools to achieve this have only recently begun to become widely available (6).

Any individual protein or signal transduction pathway exists within a hugely complex and high-dimensional cellular context as defined by the patterns and interactions among all the other proteins, metabolites, RNA, DNA, and cellular functions, operating concurrently and dynamically in the same cell. While each cell likely contains approximately 30,000 genes, estimates of the size of the human interactome vary considerably. Stumpf *et al.* estimate the human interactome (entire set of protein interactions) to be ~650,000 interactions, a sparse network of only ~0.2% of all pairwise connections (13). However, this estimate does not consider context-specific interactions or the dynamic nature of the system (13). The latter could substantially increase the number of interactions responsible for maintaining cellular function across time and in response to changing extracellular and intracellular environments. The contributions of protein-DNA, protein-RNA, and protein-metabolite/ligand interactions may not be adequately captured in this estimate and these could further increase the dimensionality of the edges in the overall signaling network regulating cellular function.

Understanding the properties of networks of this size and complexity offer remarkable challenges, not least of which are the unique properties of high dimensional spaces (10). For example, in such large networks it is estimated that the shortest distance between any two nodes (usually a gene or protein) is no longer than 6 connections (14, 15); likely a major contributor to the signaling redundancy and degeneracy that can confer apparent plasticity on network topology. Multiple inputs to the human interactome are occurring concurrently and the network is dynamically responding to each of these inputs – many of which modify the function of other regions of the interactome. This level of interconnectivity and dynamism is fundamentally lacking in most current approaches to gene network modeling. Moreover, in all likelihood, we do not yet fully understand the properties of such large networks or their implications for building fully accurate and robust models of their function.

Precisely because the human protein interactome is dynamic and adaptable, building a model of how it works has many characteristics of a “wicked problem” (16, 17). Amongst several criteria, a wicked problem is one where there is incomplete, and sometimes contradictory information, and the changing nature of the requirements of the network (in the case of a cellular system in response to stress, external signaling that may change the function or differentiation status of the cell, or other factors) that are difficult to recognize. Moreover, there may be more than one solution - what explains how the interactome works for endocrine resistance in ER+ breast cancers may not explain how it works in any other cancer resistance problems. While perhaps not all of the criteria apply, getting the scientific community to engineer an agreed solution could well be a wicked problem in the original social planning sense.

While it might be tempting to assume that these various challenges do not apply to the study of endocrine resistance, it is not immediately clear that this is a reasonable assumption (6). ER-mediated responses can encompass coordinating functions from complex organism-level sexual, aggressive and reproductive behaviors, down to the subcellular level as might be represented by coordinating the subcellular functions that are required to execute the decision of a breast cancer cell to exit G1 and enter S-phase of the cell cycle. Perhaps these functions are provided by very different ER-regulated genes in neurons, for example, than in mammary epithelial cells (the network nodes - and so also the edges - could be very different). However, nature is often parsimonious, and the possibility that many of the same molecular players in breast cells also operate in brain cells cannot be discounted. If this is the case, then it is not so much the nodes (genes/proteins) that are different in brain and breast cells, it is the edges (connections) that link them. At some level, the ER-regulated network could broadly retain its overall topology, adapting primarily (but not exclusively) by locally modifying how some of its nodes are interconnected. The same may be true for the differences between endocrine sensitive and resistant topologies of the ER-regulated network.

The current state of knowledge in biology, mathematics, statistics, and signaling transduction probably limit our ability to fundamentally address modeling of any complex biological network in a single approach. Pragmatically, we must make some general assumptions and work with the acknowledged limitations of current knowledge and existing tools. Thus, we propose that the endocrine resistance phenotype(s) is primarily controlled by a large and complex subnetwork that exists within the context of the much larger human interactome. From this starting point, a simple, linear thought process allows us to derive other reasonable but possibly incomplete assumptions about this subnetwork

In sensitive breast cancer cells, endocrine therapies initially induce a profound G_0/G_1 cell cycle arrest. Clearly, one of the regulatory functions of our hypothetical subnetwork is the decision to enter or

exit the proliferative phases of the cell cycle, and a coordinated regulation of the attendant cellular functions required to provide the energy and nutrients needed to make a duplicate copy of the existing cell. This latter series of events follows where the decision is to remain cycling and so exit G1 and enter S; as would be the case in treating resistant cells, or providing estrogen to estrogen-dependent cells.

Since endocrine therapies can lead to improvements in overall survival, at some point each breast cancer cell will make and then execute a decision to live or die. A further component of our subnetwork must govern the cell survival decision and the attendant functions that allow the cell to survive. Such functions include blocking induction of apoptotic cell death and providing for the integrity of those subcellular functions required for prolonged cell survival, such as maintaining adequate energy levels for basic metabolic functions.

ER can regulate (or at least influence) both cell proliferation and cell survival decisions, and so it must also arrange for coordinating the cellular functions required to execute these decisions. Thus, ER must be a central node in the subnetwork. Indeed, most ER+ breast cancers that acquire a resistant phenotype remain ER+ (18), and siRNA targeting ER in antiestrogen resistant cells is growth inhibitory (19). Much is known about how ER functions and of various growth factors and other signaling molecules that, in the context of endocrine regulation of breast cancer cells, can influence ER functions and endocrine responsiveness (5-7). Thus, we can begin with a simple list of genes that will become initial seed genes (nodes) around which we can build out a more complete network model (20).

Individual modules for the functional execution of the cell cycle decision are well known and these appear to have significant components maintained by evolution across multiple species. One example is the execution network that enables cells to complete a turn of the cell cycle, which was initially modeled in yeast cells (21, 22). Components of the unfolded protein response (UPR) are also conserved across species and these include homologues of X-Box binding protein-1 (XBP1). Thus, we can separate our subnetwork into a series of modules that perform specific functions, and a series of (presumably) interconnected decision signaling networks that make the determination of which execution modules to activate or repress and the timing of these execution/repression decisions. Modules would then include, at the very least, cell cycle, UPR, apoptosis, autophagy.

How we approach construction of the mathematical models and control signaling is described elsewhere (23). Overall, we apply an integrated approach where we use computational modeling tools and high dimensional data to extract local topological information of the relationships among the genes and functions we believe to be of most initial relevance. For the purposes of this review, computational modeling uses tools mostly from the field of computational statistics such as artificial neural networks

and support vector machines; these tools are used to learn the key features of the data as they relate to phenotype. By mathematical modeling we mean the process of deriving a mathematical description that captures the relevant mechanistic details of the system and can be simulated to predict how the system evolves in time. Such descriptions may, for example, use differential equations or stochastic reaction networks to model gene expression and protein interactions relevant to the phenotypes being studied. Both computational and mathematical models can generate simulations and make predictions of how the systems they are modeling responds when perturbed.

Once validated experimentally, we integrate this knowledge with preliminary mathematical models for each module and/or control function. In an iterative approach, using both computational and mathematical modeling, we begin to learn how the system may function – mostly from the failure of the initial models to recapitulate experimental data and the subsequent predictions of what functions are required to allow the models to work.

In this review, we will focus on the potential role of one module for regulating key survival functions that we have implicated in acquired endocrine resistance. Specifically, we will review evidence implicating activation of the unfolded protein response as a critical subcellular function and follow through on early computational modeling of what appears to be prosurvival signaling out from the UPR as regulated by controlling the expression and unconventional splicing of XBP1. Of necessity, these initial representations are largely static wiring diagrams. However in the longer term, we will use our experimental data and that available in the literature to guide the construction of initial mathematical models of the UPR and its role in governing prosurvival signaling in the context of endocrine responsiveness in breast cancer.

Endoplasmic reticulum stress and the unfolded protein response in normal and neoplastic breast tissues

The folding of proteins within the endoplasmic reticulum (EnR) is an energy-dependent function, which in the absence of sufficient energy or other nutrient limitations can result in the accumulation of unfolded proteins within the EnR lumen. Normally, these proteins are detected and additional energy is consumed as the cell attempts to fold (or unfold and refold) them into their correct form(s). However, as unfolded proteins accumulate, the cell may have less and less energy available to meet this increased demand, particularly if it is experiencing external stressors and the resources to fold correctly these proteins are inadequate. The accumulation of these unfolded proteins creates a condition known as endoplasmic reticulum stress, which ultimately initiates an attempt to restore

balance through several means including lowering energy/nutrient demands by reducing the rates of mRNA transcription and protein translation, and removing for degradation (rather than refolding) inappropriately folded or unfolded proteins. Degradation usually occurs through the **e**ndoplasmic **r**eticulum-**a**ssociated **d**egradation pathway (ERAD) (24). Prolonged EnR stress may activate more substantive prosurvival processes, such as a prosurvival autophagy.

Three forms of autophagy exist: microautophagy, chaperone-mediated autophagy, and macroautophagy (25) (here we use the term “autophagy” to denote macroautophagy). A lysosomal process, autophagy occurs when the cell begins to self-digest its subcellular organelles; these are usually defective organelles, perhaps rendered such by an excessive and unmet total energy/nutrient demand within the entire cell. Autophagy can be either prodeath (autophagic cell death) and act as an alternative cell death pathway to apoptosis (26), or prosurvival when extracellular nutrients or growth factors are limited (27). The primary goal of this prosurvival autophagic process appears to be to recover sufficient energy and nutrients from the unnecessary/damaged organelles to meet the demands of more fundamental cell processes.

Prolonged unresolved EnR stress often causes cell death, which may include an autophagic cell death driven by autophagy cannibalizing subcellular organelles to a point beyond which the cell can no longer survive. Whether the ultimate cell death is a consequence of induction of an energy dependent cell death process such as apoptosis, or one less dependent upon available energy sources such as necrosis, is an area of considerable interest and investigation.

The initial coordinated response to EnR stress is a process called the unfolded protein response (UPR). Since there are several excellent reviews available that describe the UPR in detail (28, 29), we here provide only a brief overview. The UPR has three primary arms, each initiated by a specific sensor, respectively PERK, ATF6, and IRE1α (Figure 1). Under normal conditions, each sensor is maintained in an inactive state through its association with the molecular chaperone HSP5A - also known as glucose-regulated protein 78 (GRP78) or immunoglobulin heavy chain-binding protein (BiP). In the presence of unfolded proteins, HSP5A disassociates from the molecular sensors and binds to the misfolded proteins in an attempt to activate their repair (30), thus activating the sensors.

It seems likely that the normal mammary epithelium has a particularly well-coordinated and active UPR. For example, the prolonged production of substantial amounts of secretory proteins is essential during lactation, when the mammary epithelial cells must balance the need to apply energy resources to translate, fold, and secrete proteins with those of the basic cell survival functions. Furthermore, it would make strong biological sense for the cell to coordinate the fulfillment of its nutrient and energy demands with this protein production requirement, so as not to induce a prolonged and potentially fatal EnR stress. Since the stimuli to regulate milk production are under the

regulation of the lactogenic hormones (prolactin, insulin, and the glucocorticoids), it is reasonable to assume that these hormones also assist in initiating and/or maintaining the coordinated functions required to balance a high rate of protein production, and the potentially associated EnR stress, with cell survival. Thus, normal breast epithelial cells are likely well primed to adapt to prolonged EnR stress, and the recruitment of these strategies by neoplastic breast cells as a primary survival mechanism in the face of the stress of endocrine therapy (or other therapies) would seem predictable. Since ER-mediated activities in breast cancer cells appear to regulate multiple functions, including general cellular metabolism and the highly energy/nutrient demanding functions required to execute a decision to enter the cell cycle, it is reasonable to expect activation of ER to play a central role in affecting UPR-associated activities in breast cancer cells.

XBP1 transcription and splicing, and its interactions with ER

The application of stress to cells results in several changes in metabolism and can induce various stress response functions. A reduction in access to adequate oxygen, nutrients, or energy can cause cells to redirect their available resources to perform basic functions in order to survive. Inadequate vascularization places many tumor cells under some level of hypoxic stress and nutrient deprivation, stressors known to induce EnR stress (31). Inhibition of ER activity in estrogen-dependent breast cancers by AEs or AIs likely exacerbates these problems and further activates endoplasmic reticulum stress. Thus, the UPR is a primary candidate for one survival mechanism that, if successfully activated, could allow cells to survive the stress of endocrine therapies and confer a resistance phenotype.

Gu *et al.* first implicated UPR signaling in antiestrogen resistance and estrogen independence, reporting the increased expression of XBP1 and its associated cAMP-response element-driven transcriptional activity and that of other UPR related proteins (NFκB; HSP27) in LCC9 breast cancer cells (32). The functional relevance of the role of XBP1 was established when the full length XBP1 cDNA was overexpressed in both the MCF-7 and T47D human breast cancer cell lines by Gomez *et al.* (33), data also consistent with the ability of the upstream UPR regulator HSP5A to protect cells from estrogen withdrawal (34). Interestingly, the primary form of XBP1 protein present is the spliced form XBP1(s), indicating that, at least in these cell models, transcriptional regulation of XBP1 may be rate limiting and not the rate of its unconventional splicing by the endonuclease activity of IRE1α. XBP1 splicing is unconventional because it occurs predominantly in the cytosol (35). While IRE1α can splice multiple RNAs, it is the only enzyme known to splice XBP1. Splicing removes a short 25 basepair

sequence from XBP1 that deletes a stop codon and creates a longer mRNA reading frame.

Translation of the XBP1(s) RNA template results in the production of a larger protein that can acts as a transcription factor. Regulation of transcription by XBP1(s) is a consequence of its homodimers activating specific cAMP response elements (CREs) with a conserved ACGT core sequence GATG**ACGT**G(T/G) NNN(A/T)T; sometimes called the UPR element (36, 37). In marked contrast, translation of the unspliced XBP(u) generates a shorter protein that cannot act as a transcription factor but can act as an endogenous dominant negative inhibitor of XBP1(s) (38, 39). Thus, consistent with the critical nature of the functions it regulates, control of XBP1 activity is multifactorial, for example, (i) rate of transcription (includes regulation by cleaved ATF6 and ER), (ii) rate of splicing by IRE1α (perhaps not a common mechanism in breast cancer), (iii) ratio of XBP(1u):XBP1(s).

Of particular relevance to breast cancer is the observation that XBP1 is a major estrogen induced gene, being rapidly induced in response to E2-stimulation (40, 41). Expression of XBP1 is a key component in the molecular classification scheme that defines luminal, basal, HER2+, and normal-like breast cancers (8), being associated with the ER+ phenotype (42). Furthermore, XBP1 protein can act as a coactivator of ER, forming ligand-independent XBP1:ER heterodimers that are more effective in driving transcription from an estrogen responsive element (43). These observations suggest that the XBP1-ER interactions may be used to “fine-tune” some critical UPR functions.

Modeling XBP1 signaling in breast cancer cells

The evidence implicating XBP1 expression in acquired resistance (32, 33) and our hypothesis of its potentially central role during lactation, led us to explore possible new predictive models of XBP1 signaling. As a precursor to developing mathematical models, we have begun to develop computational modeling tools and apply these to existing data sets to try to uncover new topological knowledge of XBP1 signaling (20, 44-47). The primary goal is to discover topological features of an XBP1-associated signaling module in the context of endocrine responsiveness, with a particular focus on an initial series of genes we believe are likely to contribute to the regulation and/or execution of proliferation or cell death/survival decisions. Subsequently, we perform wet laboratory experiments to validate and extend these topological features and to explore more fully how signaling flows to affect endocrine responsiveness. Initial models are necessarily simplistic and static in their representations of what is definitively a dynamic and adaptable process. Nonetheless, these representations should allows us to eventually build truly dynamic models that can more accurately predict the most important signaling that affects key subcellular functions relevant to the endocrine resistant phenotype. The

dynamic nature of the process is captured by the models allowing changes to be made in the input values for specific nodes or edges. The model will then calculate how the signaling is perturbed as a consequence these changed values, leading to predictions about signal transduction and the altered regulation of the relevant cellular function(s).

In our work to develop new methods for computational network modeling, we have recently developed a powerful new approach called differential dependency network (DDN) analysis (20, 48). DDN was derived specifically to model statistically significant topological changes between two conditions and was initially applied to transcriptome data from gene expression microarrays. Local dependency models decompose the whole network, as represented by the entire data set, into a series of local networks. Rather than look at 2-wise or 3-wise interrelationships, the local dependency models are applied with a Lasso technique (least absolute shrinkage and selection operator; a least squares regression method with an L1 norm constraint) that can select the optimum number of dependent variables and help ease the risk of overfitting (20, 49). To detect statistically significant network topological changes, DDN applies permutation tests under the two conditions and estimates a p-value for each of the local structures. Ultimately, local topological features are represented by a set of conditional probabilities, and each node can be assigned more than one conditional probability distribution. The latter can allow nodes to “belong” to more than one local dependency network and/or acquire multiple edges. Edges in DDN reflect the dependency structures among genes that are learned by the Lasso method. Since DDN characterizes the statistically significant network changes between two biological conditions, the dashed and solid edges in Fig 2 represent the condition-specific dependencies. For instance, if gene A is a good predictor of gene B under condition 1, but shows no such relationship under condition 2, then in the DDN we will expect there is a condition-specific edge between gene A and gene B under condition 1. A key goal of DDN modeling is to find “hot spots”, which are those genes that exhibit statistically significant network changes between two conditions given a predetermined significance level. The assumption with respect to these “hot spots” is that robust topological changes likely reflect important or meaningful biological events. Greater detail on the derivation of this method can be found elsewhere (20, 48).

In our initial studies, we selected 55 genes associated with antiestrogen responsiveness, including XBP1, and applied DDN to a publicly accessible gene expression microarray data set from T47D human breast cancer cells treated with 17β-estradiol ± Fulvestrant (Faslodex; ICI 182780). Fulvestant is an ER antagonist antiestrogen that does not exhibit partial agonism and normally targets the ER for degradation (50). The study from which the data were obtained was reported in detail by Lin *et al.* (51), and incorporates time course experimental design of 16 time points over a 24 hr period. Thus, we used DDN to look for topological features in the data set that could reflect “early” estrogen

regulated signaling that is perturbed by the antiestrogen.

Initial representation of XBP1-associated signaling

The results of these initial studies using DDN are shown in Figure 2; this is a general representation of one small area of the overall subnetwork regulating the cell fate decision and provides a series of seed nodes and edges for validation in wet laboratory experiments (20, 48). The edges are coded to reflect those present with E2 treatment (solid lines) and those present with estradiol and Fulvestrant co-treatment (dashed lines). Hence, solid lines are implied to disappear when the antiestrogen is added. From the perspective of XBP1, proposed connections with BCL2 and NFkB would be present only with estrogens and lost with the addition of Fulvestrant. BCL2 is a key determinant for maintaining cell survival with the UPR (28), and we now know that BCL2 is overexpressed in antiestrogen resistant cells that also overexpress the endogenous XBP1(s) (52). Estrogenic induction of BCL2 is well known, and we have shown that XBP1 is also a likely regulator of BCL2, which is overexpressed in cells that have been transfected with XBP1 (33). The BCL2 promoter contains at least three XBP1-CRE sites that could drive a direct transcriptional activation of BCL2 by XBP1 (Figure 3A). When considered together, these data strongly suggest that some breast cancer cells may use the cooperation between ER and XBP1 to provide redundant signaling and increase the likelihood of cell survival despite any concurrent EnR stress (Figure 3B). Importantly, antiestrogen resistant cells that overexpress BCL2 are more sensitive to growth inhibition by small molecule inhibitors of BCL2 (52).

The DDN model already correctly incorporates known knowledge of the relationship between XBP1 and BCL2. New relationships are predicted including potential roles for ERβ (ESR2), BCAR3, and NFkB. Data implicating each of these genes *individually* in antiestrogen responsiveness is already available. For example, NFKB2 is associated with estrogen independence and may be selectively activated in breast tumors (53). BCAR3 activity is strongly associated with estrogen independence, and antiestrogen resistance (54-56). This small topological representation includes two MAPK family members (MAPK3; MAK13), suggesting that it also may begin to explain coordinated signaling for the regulation of both proliferation and survival. Most of the edges represented in Figure 2 remain to be experimentally validated, and whether there are intervening latent variables is unknown at this time. Nonetheless, the model provides further evidence implicating UPR associated genes in endocrine responsiveness and offers some novel hypotheses as to how these genes may further interact.

The implication that BCL2 is a key player may represent more than this single gene - the model could also be read as implicating its function; as such, the role of BCL2 in Figure 2 could reflect

a role for several members of this family. We have recently shown that the full effect of the small molecule BCL2 inhibitors is mimicked only when both BCL2 and BCL-W are co-inhibited (52). Other interactions also occur but these are not directly reflected in this model. For example, BCL2 and BCL-W can affect cell survival by binding and sequestering BECN1. These interactions prevent the induction of a prodeath autophagy and can contribute to antiestrogen resistance (52). However, these events are further downstream and occur primarily in the proteome, and so might not be reflected in a model based primarily on transcriptome data. This observation identifies one limitation to using such models to try to solve an entire subnetwork topology. However, to understand the transcriptional components of the subnetwork, the application of DDN to gene expression microarray data can uncover known relationships and propose new hypotheses for further study.

How the full subnetwork regulating endocrine resistance is wired remains unknown. Nonetheless, the extraction of topological information supported by experimental biological data in relevant cell systems provides a starting point from which to uncover new nodes and edges and build out the network in an iterative manner (6). As we obtain additional experimental data, we can eventually move towards constructing mathematical representations of the signaling and network function. Ultimately, we will build predictive models that capture how ER-mediated signaling coordinates cell survival and cell proliferation decisions, and the required metabolic and other cellular functions that must be activated or repressed to execute these functions.

Conclusions and future directions

In estrogen dependent cells, estrogen withdrawal (AI) or ER blockade (AE) results in a loss of adequate metabolic activity, likely resulting in low energy production. Inadequate energy depletes exiting stores and eventually fails to meet the needs of the EnR to fold new proteins. This chain of events results in activation of an endoplasmic reticulum stress and induction of the UPR in an attempt to rebalance the energy and nutrient demands the cells need for survival. Those cells best capable of adapting their prosurvival signaling will have the greatest probability of acquiring a stably resistant phenotype. Where this signaling involves upregulation of XBP1(s), the cells will also have a greater likelihood of becoming crossresistant to other endocrine therapies. UPR initiated signaling may also result in an upregulation of autophagy, with surviving cells being those that can adjust this self-digestion to balance the need for energy and nutrients with the risk of activating cell death cascades. A critical signaling integration point for these activities appears to include modulation of the expression of various members of the prosurvival BCL2 family including, but not limited to, BCL2, BCL3, and BCLW

(52, 53, 57, 58).

The need to develop a greater understanding of the signaling that regulates endocrine responsiveness is evident. While much is known about the potential contribution of individual genes, and perhaps also some relatively linearly constructed signaling pathways, how this knowledge can be used to build dynamic, predictive models of cell function remains elusive. To create more effective combinatorial therapies it is likely that we must understand the topology of the network with sufficient clarity that we can target only those nodes/edges needed to cause the signaling to collapse, and for the cell to have the least chance to adapt or rewire its signaling to survive. If we are correct, the current practice of treating ER+ breast cancers with single agent endocrine therapies may eventually be replaced with modalities that are more complex. Among the challenges in arriving at this point will be obtaining an adequate understanding of signaling complexity, being able to model the inherent redundancy and degeneracy naturally present within networks that control and execute such fundamental decisions (and that contribute to the apparent plasticity of the phenotypes), and developing safe and effective new drugs for these targets. While this is very probably a wicked problem, current approaches to ease the challenges for this problem include the integration of mathematical and computational tools to help guide the modeling and offer hypotheses for the laboratory experimentalists to test. Data from the hypothesis-testing laboratory experiments provide further insights to adjust iteratively computational and mathematical models. In addition to the need to apply some standard reductionist wet laboratory experiments, at least for the time being, high throughput experimental methods such as the various microarray, proteomic, sequencing, and functional genomics tools now available offer the opportunities to obtain much of the data required to eventually allow building useful models.

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Figures

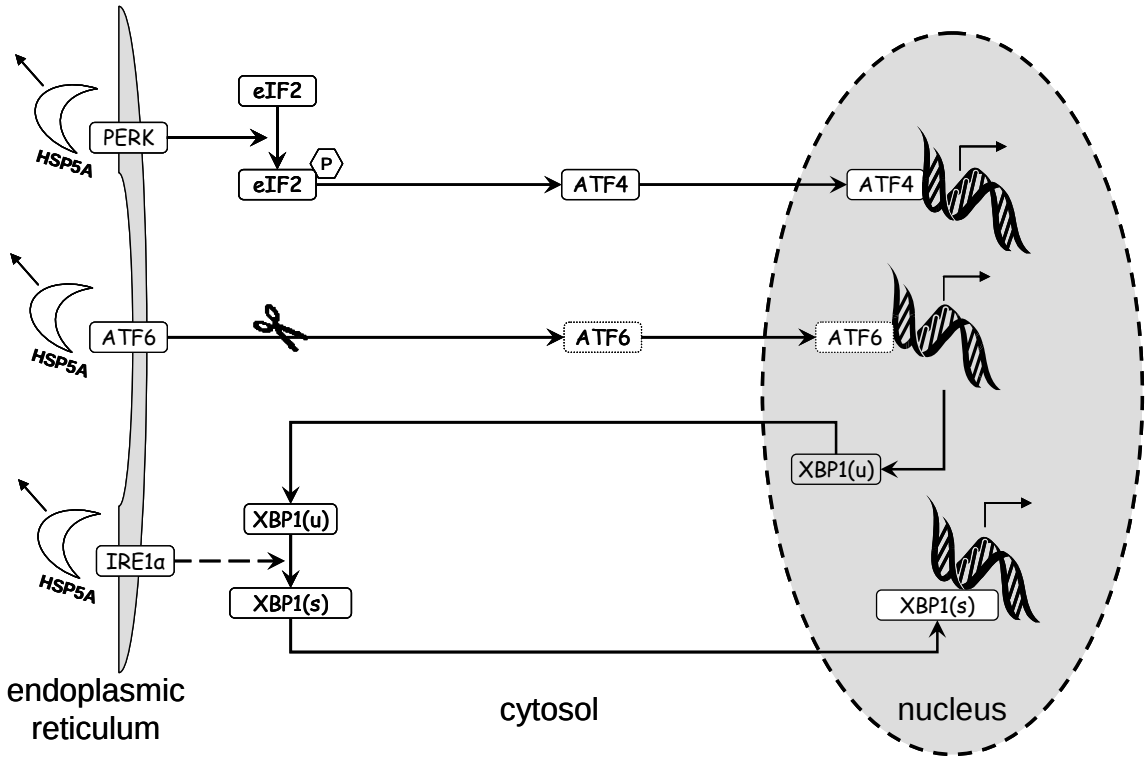

Figure 1 Overview of the three arms of the unfolded protein response (UPR). Accumulation of unfolded proteins causes HSP5A to dissociate from (and so activate) the three sensors PERK, ATF6, IRE1α. The activated sensors then initiate their respective signaling arms, each of which results in the regulation of transcription (by ATF4, cleaved ATF6, and spliced XBP1, respectively). The role of XBP1 in the ATF6 arm (induction of XBP1(u) transcription) and IRE1α arm (creation of XBP1(s) by XBP1(u) splicing) is shown.

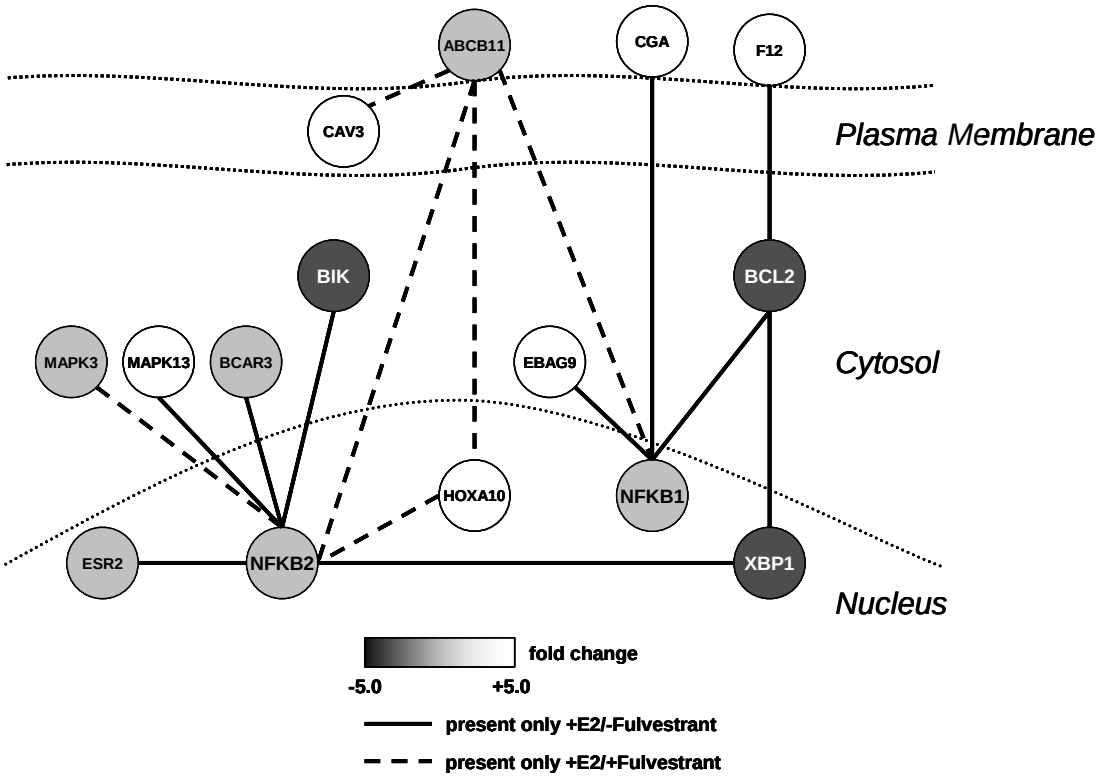

Figure 2 DDN model showing initial topology (nodes and predicted edges) of a subnetwork featuring XBP1, BCL2, and NFκB. Overexpression of XBP1 in MCF-7 cells results in the upregulation of endogenous BCL2; the BCL2 gene has three XBP1-CREs in its upstream promoter region. Solid edges are those present with E2 treatment; dashed edges are present with 17β-estradiol and Fulvestrant co-treatment. Adapted from figure 3 in reference (20).

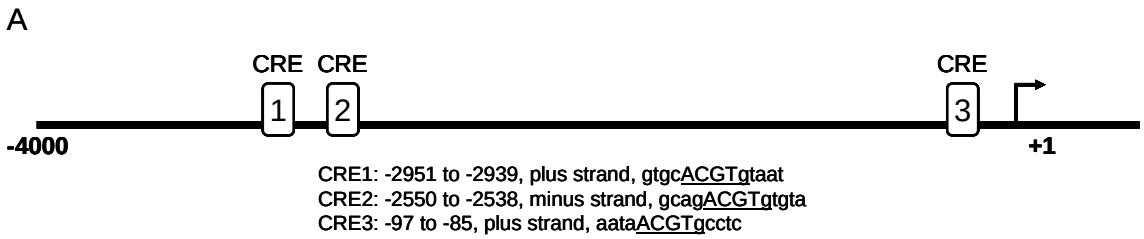

Figure 3A BCL2 promoter contains at least three of the specific cAMP responsive element sequences regulated by XBP1(s).

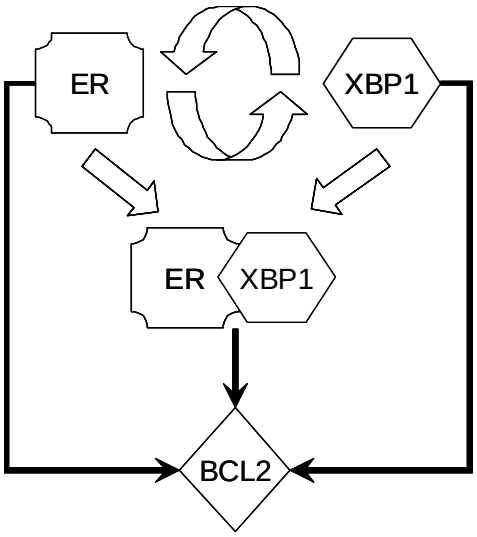

Figure 3B ER and XBP1 interact to induce the prosurvival factor BCL2. ER can induce XBP1, and XBP1 can induce ER. ER and XBP1 can form transcription complexes that are more effective at driving transcription from EREs. Independently, ER and XBP1 (and presumably also ER:XBP1 complexes) can induce BCL2, providing integrated and potentially redundant prosurvival signaling from the UPR.

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Cancer Research

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Review

Cancer Research

Endoplasmic Reticulum Stress, the Unfolded Protein Response, Autophagy, and the Integrated Regulation of Breast Cancer Cell Fate

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Abstract

How breast cancer cells respond to the stress of endocrine therapies determines whether they will acquire a resistant phenotype or execute a cell-death pathway. After a survival signal is successfully executed, a cell must decide whether it should replicate. How these cell-fate decisions are regulated is unclear, but evidence suggests that the signals that determine these outcomes are highly integrated. Central to the final cell-fate decision is signaling from the unfolded protein response, which can be activated following the sensing of stress within the endoplasmic reticulum. The duration of the response to stress is partly mediated by the duration of inositol-requiring enzyme-1 activation following its release from heat shock protein A5. The resulting signals appear to use several B-cell lymphoma-2 family members to both suppress apoptosis and activate autophagy. Changes in metabolism induced by cellular stress are key components of this regulatory system, and further adaptation of the metabolome is affected in response to stress. Here we describe the unfolded protein response, autophagy, and apoptosis, and how the regulation of these processes is integrated. Central topologic features of the signaling network that integrate cell-fate regulation and decision execution are discussed. *Cancer Res; 72(6); 1321–31. ©2012 AACR.*

Introduction

Cell fate primarily involves a cell's decision to live or die. If the decision is to live, the cell must then decide whether to differentiate, arrest growth, or enter the cell cycle. If the decision is to die, the cell must activate a programmed cell death (PCD) pathway such as apoptosis (PCD1), autophagy (PCD2), or necrosis (PCD3). Appropriate regulation of these cell-fate decisions is often critical during normal development, tissue differentiation, and response to stress. The breast provides a useful example of these processes. Normal breast function includes periods of proliferation and differentiation in preparation for lactation, followed by the PCD that occurs during involution as the postlactational breast returns to a resting state. Inappropriate activation/repression of cell-fate decisions can have major consequences, and the loss of regulation of cell cycling, as well as inappropriate cell survival, are common characteristics of neoplasia. How cells integrate complex cell-fate signals, and whether this process differs between normal and neoplastic breast cells, remains unclear. For example, during lactation, the normal breast must balance the extensive production of milk proteins with the risk that an excessive load of these proteins could result in endoplasmic reticulum (ER) stress and induction of the unfolded protein response (UPR). In breast tumors, stress that induces a UPR can arise from the nutrient deprivation and hypoxia induced by inadequate vascularization and from the application of cytotoxic and endocrine therapeutic interventions. Because the UPR can be either prodeath or prosurvival, both the lactating and neoplastic breast must maintain a prosurvival UPR, perhaps using many of the same regulatory mechanisms.

Signaling initiated within the UPR leads to changes in the levels and activities of key regulators of cell survival, with the integration of both prodeath and prosurvival signals and functions determining cell fate. Determinants in this process include signals that cross-talk among the plasma membrane, ER, mitochondria, cytosol, and nucleus, leading to the eventual induction or repression of apoptosis and/or autophagy, and the changes in cellular metabolism that are necessary to enable execution of these decisions. In the breast, the central molecular players in this orchestration include members of the B-cell lymphoma 2 (BCL2) and autophagy-related (ATG) gene families, estrogen receptor-α (ERα, ESR1); nuclear factor κB (NF-κB, RELA), and components of the UPR, such as X-box binding protein-1 (XBP1) and its unconventional splicing.

Precisely how cancer cells die following either endocrine or cytotoxic interventions is unclear; however, several

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independent but potentially interrelated cell-death mechanisms are known (Fig. 1). For example, mitotic catastrophe may be important in response to therapies that target microtubules (1). For endocrine therapies, the extent to which necrotic cell death occurs is uncertain (2, 3), but cell death by apoptosis (4–7) and autophagy (8, 9) *in vitro* are consistently reported.

Emerging evidence is beginning to define a more intimate relationship between apoptosis and autophagy, implying significant communication between these 2 activities. Such communication may reflect the use of similar or related signaling molecules in an integrated or even interdependent manner. For example, events within the mitochondria and ER, and their regulation by BCL2 family members, are areas of commonality in apoptosis and autophagy (10, 11).

ER stress and the UPR

The rates of protein synthesis and secretion are tightly linked to the ability of the ER to fold, process, and traffic newly synthesized proteins. Within the ER, nascent proteins are appropriately folded and moved to the Golgi apparatus for

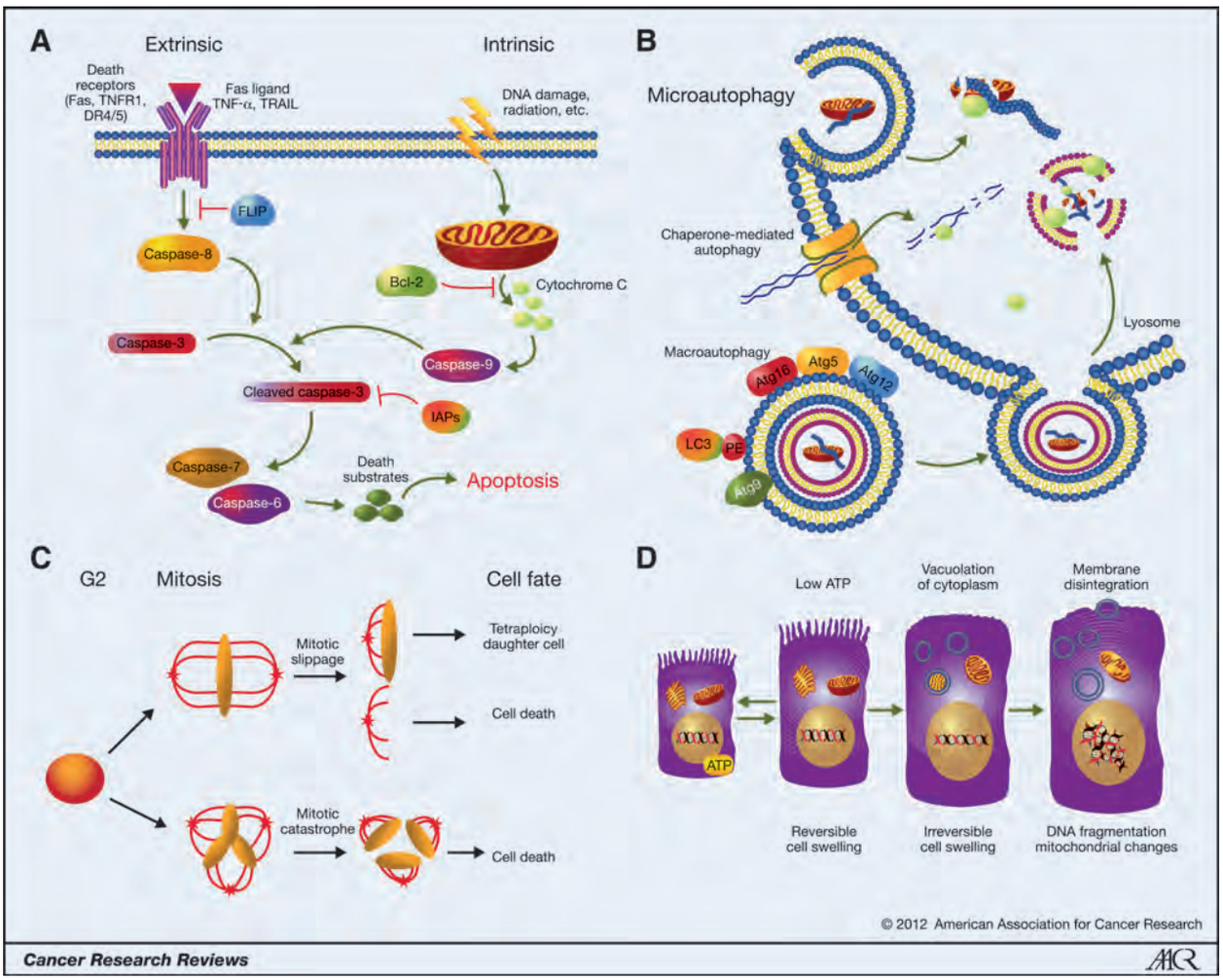

Figure 1. Mechanisms of PCD. A, apoptosis is an ATP-dependent process characterized by organized chromatin condensation and fragmentation of the nucleus, DNA cleavage, formation of apoptotic bodies, cell shrinkage, and plasma membrane ruffling (103–105). The intrinsic (mitochondrial) pathway is regulated by BCL2 family members and involves changes in mitochondrial membrane permeability, release of cytochrome c, exposure of phosphatidylserine, and loss of plasma membrane integrity (106). The extrinsic (cell surface receptor) pathway is dependent on extracellular signals, including TNF-α, Fas ligand, and the TNF-related ligand TRAIL (104, 105). B, the 3 forms of autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy) involve the degradation of cellular contents by lysosomal hydrolases. Macroautophagy (the focus of this review) requires the formation of double-membrane structures called autophagosomes or autophagic vacuoles (107), for which accumulation of autophagosomes and cleavage of the microtubule-associated protein LC3 are characteristic but often not definitive (47). C, mitotic catastrophe produces multinucleation or the products of micronuclei. Faulty checkpoints, DNA structure checkpoints, and the spindle assembly checkpoint are key components (108, 109). Disruption of the normal segregation of many chromosomes results in rapid cell death (108). When cell death does not occur, the cell can divide asymmetrically and produce aneuploid daughter cells (110) that can become neoplastic (108, 110). D, necrosis is induced when the intracellular concentration of ATP falls to a level that is incompatible with survival (111). Vacuolation of the cytoplasm, breakdown of the plasma membrane, and induction of inflammation around the dying cell are characteristic (45). DNA fragmentation and increased membrane permeability in the absence of organized chromatin condensation also occur (45, 112). Increased cell volume causes rupturing of the plasma membrane and the disorganized breakdown of swollen organelles.

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further trafficking. Folding of the polypeptide chain is achieved through the actions of a series of molecular chaperones and foldases, which keep the polypeptide in solution and facilitate folding of the chain into a thermodynamically favored structure. When this process is incomplete, the cell must deal with any proteins or protein subunits that remain unfolded or misfolded within the ER, which can become characteristically distended (ER stress). If this is unresolved, protein folding becomes further impaired because inappropriately folded proteins continue to sequester molecular chaperones and activate their ATPases. Continual disulfide bond reduction and reformation depletes both energy and reducing molecules such as glutathione, increases the generation or persistence of reactive oxygen species, and creates oxidative stress, further damaging the existing proteins and further limiting their appropriate folding.

Up to one third of cellular proteins are synthesized within the ER (12). To address the adverse effects of accumulating unfolded proteins, the cell induces a series of events collectively known as the UPR (ER stress response; Fig. 2A). The primary goal of the UPR is to eliminate inappropriately folded proteins and reduce the load of newly synthesized unfolded proteins within the ER. It accomplishes these actions by reducing the amount of mRNA template for proteins by degrading existing mRNAs, slowing the transcription/translation of new mRNA, and reducing the influx of nascent proteins into the ER lumen (13). Concentrations of protein folding effectors, including molecular chaperones and foldases, are also increased to process the mass of accumulated proteins. The remaining misfolded proteins are eliminated through one of 2 ER-associated degradation (ERAD) pathways (14): a ubiquitin/proteasome pathway known as ERAD(I) or an autophagic/lysosomal pathway known as ERAD(II) (15). Soluble targeted proteins are retrotranslocated into the cytosol, ubiquitinated, and then degraded by the proteasome in ERAD(I) (16, 17). Insoluble misfolded protein aggregates are degraded by autolysosomes in ERAD(II) (15, 18).

Accumulation of unfolded or misfolded proteins is detected by ER transmembrane receptors. The 3 primary molecular sensors are inositol-requiring protein-1α (IRE1α, ERN1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase [PERK, EIF2AK3 (19)]. In the absence of stress, each is maintained in an inactive state through its association with glucose-regulated protein 78 (GRP78; BiP; HSPA5). As unfolded proteins accumulate, HSPA5 dissociates from the molecular sensors and binds to hydrophobic domains on the surface of these unfolded proteins (20) in an attempt to effect their repair (21). All 3 arms of the UPR can be regulated by changes in the concentration of free HSPA5 (Fig. 2A; ref. 22), but how this leads to stress-specific activation of selected UPR signaling is uncertain (19).

PERK signaling in the UPR

Some UPR-associated signaling may not be unique to the UPR. Three signaling processes have been suggested: (i) signaling through IRE1α/XBP1 and ATF6 that is largely restricted to the UPR; (ii) signaling through PERK and eukaryotic translation initiation factor-2α (eIF-2α, EIF2S3) that can be restricted to the UPR; and (iii) signaling through PERK/eIF-2α and ATF6 that may be specific to the UPR but can also be induced by other stressors (Fig. 2A; ref. 23). Activation of PERK signaling appears to be independent of signaling that involves either ATF6 or IRE1α (23), and may be the least distinctly definitive pathway of the UPR. For example, the primary target of PERK (eIF2α) is also activated by protein kinase RNA-activated (PKR), eukaryotic translation initiation factor-2α kinase 4 (EIF2AK4), and eukaryotic translation initiation factor-2α kinase 1 [EIF2AK1 (24)]. Recent studies indicate that protein kinase B (AKT) phosphorylates and inhibits PERK (25). AKT-mediated inhibition of PERK signaling can inhibit the downstream phosphorylation of eIF2α, preventing the cytoprotective activity of eIF2α. Inhibition of the PERK/eIF2α pathway leads to increased cell death in tumor cells in response to phosphoinositide 3-kinase (PI3K) and AKT inhibitors, indicating a possible role of PERK/eIF2α signaling in PI3K/AKT inhibitor resistance (25). Together, these observations suggest a prosurvival role of PERK/eIF2α signaling in UPR. PERK signaling inhibits translation to reduce the protein load on the ER and increases p53 levels through a PERK-required ribosomal-Hdm2 interaction, preventing Hdm2-mediated p53 ubiquitination (26). Increases in p53 in response to UPR activation lead to cell-cycle inhibition, suggesting another adaptive method for UPR-mediated cell survival.

IRE1 and XBP1 signaling in UPR

Details about how the balance between prodeath and prosurvival UPR outcomes is determined are only beginning to emerge. Using mathematical modeling, Rutkowski and colleagues (27) proposed a model in which the prosurvival outcome is driven by the relative stability of the UPR mRNAs and proteins associated with the restoration of metabolic homeostasis, balanced by the relative instability of molecules that promote apoptosis. Lin and colleagues (28) showed that ER stress activates both prosurvival and prodeath signaling, with the outcome determined by the maintenance (prosurvival) or termination (prodeath) of IRE1α activity.

When the key activity within prosurvival UPR signaling is the duration of IRE1α activation (28), cell-fate outcome is substantially mediated by the unconventional splicing of XBP1 (19, 29), one of the primary regulators of the transcription network activated by the UPR (30). Conventional mRNA splicing generally occurs within spliceosomes in the nucleus. Non-spliceosomal extranuclear splicing can occur when essential components of the spliceosome are present, such as in the cytoplasm of platelets (31). Unconventional splicing occurs in the cytoplasm and is largely independent of spliceosomal components. For XBP1, this splicing is accomplished by the endoribonuclease activity of IRE1α. Splicing removes a 26 bp sequence (Fig. 2B), creating a frameshift that encodes a larger protein, XBP1(S), that can now act as a transcription factor. Regulation of transcription by XBP1(S) is a consequence of its ability to activate specific cyclic AMP response elements (CRE) with a conserved ACGT core sequence (32, 33). XBP1(S) can also regulate transcription from ER stress response elements [ERSE1, consensus sequence CCAAT-N9-CCACG (34)]. The unspliced mRNA protein product, XBP1(U), has a molecular

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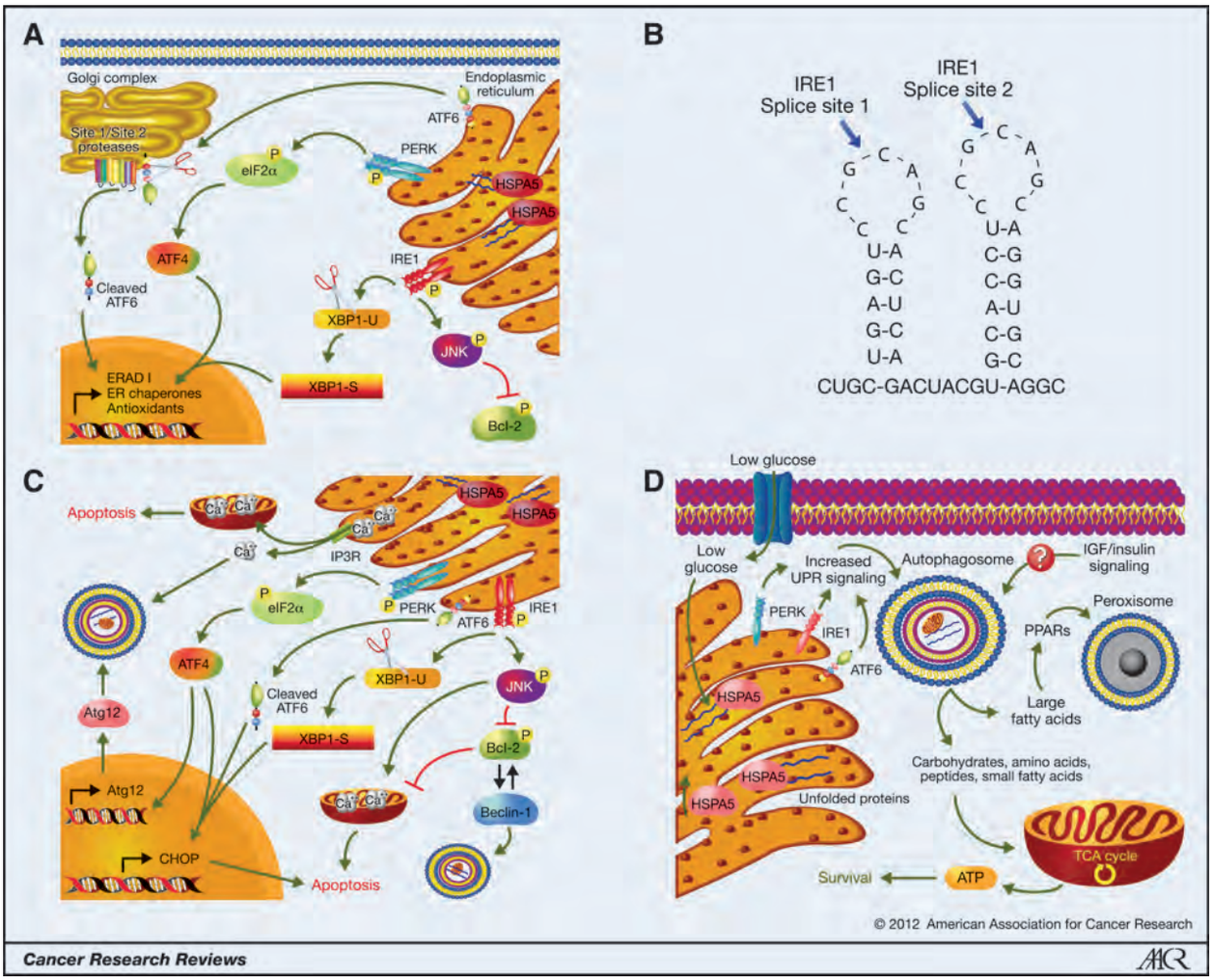

Figure 2. UPR and cross-talk between apoptosis and metabolism. A, the UPR is an adaptive signaling pathway in which the proximal activators of each of its 3 arms (PERK, ATF6, IRE1α) are normally activated following their dissociation from HSPA5 (GRP78, BiP). When released from HSPA5, the N-terminal luminal domains of 2 PERK proteins bind together (113). The resulting dimer undergoes an activating autophosphorylation, and phosphorylation of PERK tyrosine-615 is a key event (114). PERK is a type I transmembrane protein that phosphorylates eIF2α (115). Phosphorylation of eIF2α at serine-51 blocks translational initiation (116) because, as a dominant negative inhibitor of eIF2β, the EI2F recycling required for further protein biosynthesis is blocked and the rate of protein biosynthesis is reduced (114). Downstream events include induction of ATF4, which then regulates the expression of several genes, including the proapoptotic DDI3 [also known as CHOP or GADD153 (117)]. The 2 mammalian ATF6 alleles (ATF6α and ATF6β) encode a type II transmembrane bZIP transcription factor. HSPA5 blocks 2 Golgi localization signals that are exposed upon its dissociation from ATF6 (118). Following translocation to the Golgi, regulated intramembrane proteolysis by S1P and S2P cleaves, ATF6α to its active p50 form (ATF6β plays only a minor role in the UPR). ATF6 p50 then enters the cytosol, translocates to the nucleus, and activates transcription in cooperation with the general transcription factor NF-Y (119, 120). The key genes regulated by ATF6 p50 include XBP1 [spliced in the IRE1α pathway (121)], DDI3 [also induced in the PERK pathway (122)], and HSPA5 [regulates all 3 pathways (123)]. Activation of IRE1α and splicing of HAC1 (yeast) and XBP1 represent the oldest and most conserved pathway for UPR signaling. Downstream targets of XBP1(S) include $p58^{IPK}$ and several UPR chaperones (124). $p58^{IPK}$ represses PERK activity (125). Thus, persistent XBP1(S) production in the face of continued ER stress could shift UPR signaling from PERK to favor IRE1α and/or the integration of ATF6 (though increased XBP1 transcription) and IRE1α (through increased XBP1 splicing) signaling. B, 2 stem-loop structures, each containing a highly conserved CNGNNG motif, are cleaved. How the 2 exons are ligated in mammalian cells remains unclear, and this function likely differs from that described for yeast (126). XBP1 splicing may not be exclusively cytosolic, but this remains controversial (127). Other nucleotide substrates may exist for IRE1α, but none are known to possess the stem-loop structures evident in XBP1 and HAC1. C, UPR modulates cross-talk between autophagy and apoptosis through various mechanisms. Stimulation of UPR results in an increase in CHOP that promotes apoptosis. Moreover, IRE1α activation promotes apoptosis by phosphorylation of JNK, directly and indirectly inactivating antiapoptotic BCL2 proteins. UPR release of ER Ca^{2+} also directly promotes apoptosis. UPR signaling also stimulates autophagy. Activation of PERK and the resulting phosphorylation of eIF2α promote autophagy through ATF4-mediated Atg12 transcription. Furthermore, IRE1α-mediated activation of JNK and the subsequent phosphorylation of BCL2 result in dissociation of the BCL2/BECN1 complex, promoting autophagy. D, low intracellular glucose concentrations result in the accumulation of unfolded proteins, stimulating the release of the 3 UPR signaling arms (PERK, IRE1, and ATF6) by HSPA5 and activating the UPR. UPR signaling can activate autophagy, resulting in increased degradation of cellular material and the release of peptides, amino acids, and fatty acids. PPARs likely play a major role during metabolic stress by ensuring adequate turnover of peroxisomes to manage the greater metabolic requirement for release of the energy stored in the longer-chain fatty acids. Autophagy degradation byproducts (amino acids, carbohydrates, and short-chain fatty acids) promote the tricarboxylic acid cycle and the corresponding generation of ATP. Formation of ATP by mitochondria, using the raw material provided by autophagy, enables the cell to cope with low glucose levels and promotes survival. IGF, insulin-like growth factor; S1P, site-1 protease; S2P, site-2 protease.

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mass of ~33 kDa and can act as a dominant negative of the spliced XBP1(S) mRNA protein product that encodes a protein of ~54 kDa (35, 36). Activation of both ATF6 (induces XBP1 transcription) and IRE1α (splices XBP1) can be coordinated by their respective dissociation from HSPA5. This coordinated activation, and the eventual balance between the relative production of XBP1(U) versus XBP1(S), could have significant consequences for UPR activation, function, and cell fate.

In addition to increased XBP1 transcription by the UPR (30), XBP1 is also rapidly induced in breast cancer cells following 17β-estradiol (E2) stimulation (37, 38). Upregulation of XBP1 by activation of the UPR or by a UPR-independent mechanism confers antiestrogen resistance and implicates XBP1 function as an important component of breast cancer signaling (39). Moreover, expression of XBP1 mRNA is strongly associated with ESR1 positivity in breast tumors (40), and XBP1 can bind to and activate ESR1 in a ligand-independent manner (41). XBP1(S) expression is associated with acquired endocrine resistance (42). Overexpression of XBP1 cDNA in breast cancer cells produces primarily XBP1(S) and is sufficient to confer both E2 independence and antiestrogen cross-resistance (39). Expression of XBP1(S) is elevated in breast tumors that respond poorly to tamoxifen (43).

UPR and the regulation of autophagy and apoptosis

The UPR regulates multiple signals in an attempt to restore metabolic homeostasis, a process that could be fruitless if the cell did not concurrently attempt to block cell-death signaling long enough to determine whether the stress could be adequately resolved. The most effective means of accomplishing both tasks would be to integrate their respective signals. This integration can be initiated within the UPR and yet concurrently regulate both autophagy and apoptosis.

Autophagy (macroautophagy) is a lysosomal degradation process in which cellular components are encapsulated within autophagosomes and degraded by lysosomal hydrolases [see Cook and colleagues (10) for a recent review]. The signaling network topology associated with autophagy is complex and only beginning to emerge (44). Autophagy is generally characterized by the presence of cytoplasmic vacuoles and autophagosomes, the absence of marginated nuclear chromatin (45, 46), an increase in cleavage of microtubule-associated protein 1 light chain 3 (LC3), and a reduction in p62/sequestosome-1 (p62/SQSTM1) protein levels (47). LC3 cleavage, which can require eIF2α phosphorylation by PERK within the UPR (48), may not occur with noncanonical ATG5/ATG7-independent autophagy (49). Under normal conditions, basal autophagy removes long-lived proteins and damaged organelles, releasing the degradation products into the cytosol as intermediate metabolites. Autophagic removal of specific organelles [e.g., pexophagy (peroxisomes), mitophagy (mitochondria), crinophagy (Golgi), ribophagy (ribosomes), and reticulophagy (ER)] is uniquely identified.

The level and duration of autophagy can vary significantly, and, like the UPR, autophagy is associated with both cell survival and cell death (33). Prosurvival autophagy likely depends on recycling of cellular contents to feed the cell's basal metabolic machinery at a level sufficient for survival. An induction or persistence of autophagy, such that the minimum subcellular machinery necessary for survival is no longer maintained, could result in autophagic and/or apoptotic cell death. Prodeath outcomes may reflect the need to eliminate cells that cannot function normally due to the absence of key proteins, have failed to secrete correctly folded proteins (including essential hormones and growth factors), and/or have been subjected to excessive or irreversible oxidative stress and DNA damage (33).

Beclin-1 and BCL2 interactions determine activation of autophagy

Two primary regulatory activities have been reported to initiate autophagosome production in canonical autophagy signaling (50, 51). Beclin-1 (BECN1) acts through its ability to form the BECN1 complex, which includes PI3K class 3 (PI3KC3), Vps34, and Vps15, and activating molecule in BECN1-regulated autophagy (AMBRA1; Fig. 1). Alternatively, derepression of ULK1 (ATG1) by suppression of mTOR (10) or phosphorylation by AMP kinase [AMPK (52)] enables the formation of a protein scaffold to build the preautophagosomal structure. Signaling initiated within the UPR can affect both of these autophagy-initiating mechanisms.

BECN1 binds to and is inhibited by BCL2, BCL-X_L (BCL2L1), BCL-W (BCL2L2), and MCL1. Proteins that regulate the expression and/or interact with these BCL2 family members affect their ability to inhibit BECN1's proautophagic function. Thus, competitive interactions by BAD, BID, BIK, BIM (BCL2L11), BNIPL, BNIP3, NOXA (PMAIP1), and PUMA (BBC3) can promote autophagy by effectively sequestering BECN1 inhibitors and releasing free BECN1 to act elsewhere (10). Phosphorylation of BECN1 by death-associated protein kinase (DAPK) reduces BECN1's affinity for BCL-X_L (53), also releasing BECN1. Subcellular localization is critical. BCL2 inhibition of BECN1 is evident in the ER, but not when this interaction occurs at mitochondria (54). The apparent ability of BCL2 to sequester AMBRA1 at mitochondria can prevent formation of the BECN1 complex at the ER, whereas BCL2 cannot bind AMBRA1 when they are localized in the ER (55). Once autophagy is initiated, AMBRA1 can cause BCL2 to dissociate from BECN1 (55), perhaps reflecting the binding of BCL2 and BECN1 at distinct sites on AMBRA1 (56). The relative importance of location for the action of other BECN1-interacting proteins requires further clarification.

Other key regulatory events can be initiated within the UPR and directly affect autophagy, including the ability of XBP1(S) to transcriptionally induce BCL2 expression (39). Given the importance of IRE1α in affecting UPR prodeath/prosurvival outcomes (28), and by implication the importance of XBP1 splicing, the ability of XBP1(S) to regulate BCL2 expression may be one of several essential downstream activities that integrate UPR and autophagy signaling. For example, endogenous XBP1(S) is overexpressed in antiestrogen-resistant breast cancer cells (42), and its overexpression increases BCL2 expression and induces antiestrogen resistance in sensitive cells (39). BCL2 inhibition can partly reverse XBP1-induced antiestrogen resistance, but a greater effect is seen when both BCL2 and

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BCL-W are inhibited, and a further improvement is seen when BECN1 is also inhibited by either 3-methyladenine or anti-BECN1 shRNA (57). XBP1 can bind ESR1 and increase its transcriptional potency (39, 41). Because ESR1 can also induce BCL2 expression, XBP1 can potentially drive BCL2 through 2 independent mechanisms (directly through ACTG-CRE sites in the BCL2 promoter or indirectly through ESR1), providing redundancy for XBP1 regulation of BCL2 (58).

NF-κB has multiple functions, including regulation of the inflammatory response and apoptosis. Because ER stress is associated with increased production of reactive oxygen species and oxidative stress, it is logical that NF-κB and its signaling would be activated. In the context of UPR, NF-κB can be activated by PERK through the action of phosphorylated eIF2α and its regulation of IκBα translation (59). In some cells, NF-κB can induce BECN1 expression (60). NFκB can inhibit CHOP (GADD153) and prevent ER-induced cell death, establishing a link between NFκB and UPR regulation (61). Of importance, endogenous NFκB expression is increased in antiestrogen-resistant breast cancer cells, in part through the increased expression of p65/RELA and IKKγ [IKBKG (42, 62)]. Activation of PERK may contribute to increased NF-κB activity, and overexpression of XBP1(S) also increases endogenous NF-κB transcription and activation in breast cancer cells (R. Hu, et al.; unpublished data). Activation of NFκB increases BCL2 expression, and inhibition of either NF-κB (62) or BCL2 (57) can partly restore antiestrogen sensitivity in resistant cells.

c-*Jun*-NH_2-kinase (JNK, MAPK8) is activated following the binding of IRE1α and TNF receptor-associated factor 2 (TRAF2), a process that often requires signal-regulating kinase-1 [ASK1, MAP3K5 (63)]. ASK1 is strongly implicated in ER stress-induced autophagy, a process that is accompanied by IRE1α activation (64). Phosphorylation of BCL2 by JNK does not affect BCL2 binding to AMBRA1 (55), but it can disassociate BCL2 from BECN1, potentially freeing BECN1 to initiate autophagosome formation. Although JNK has roles in both intrinsic and extrinsic apoptotic pathways (65), basal levels of JNK and phospho-JNK expression are increased in antiestrogen-resistant cells (66), suggesting that JNK plays a dominant role in prosurvival UPR/autophagy rather than in apoptosis. These activities may reflect the release of BCL2 (antiapoptotic) and BECN1 (prosurvival autophagy) from each other.

UPR, autophagy, and apoptosis pathway crosstalk

Many of the UPR signaling outputs associated with autophagy are also associated with the regulation of apoptosis (Fig. 2C). For example, NF-κB and JNK activation contribute to the regulation of apoptosis. Both caspase-8 and apoptosis are activated when NF-κB activity is inhibited in antiestrogen-resistant breast cancer cells, whereas autophagy is not (66). NF-κB can directly regulate BCL2 expression, which partly explains NF-κB's ability to influence both autophagy and apoptosis. Antiapoptotic BCL2 action in the mitochondria is well known, and the binding between AMBRA1 and BCL2 at mitochondria is reduced during apoptosis (55). Association of IRE1α with BAK and BAX likely also affects apoptosis (67), and the loss of IRE1α activation enables the induction of apoptosis (28). Indeed, many members of the BCL2 family, including those implicated above in sequestering BECN1-interacting proteins, are intricately involved in the functional regulation of apoptosis (11).

Antiestrogens induce both apoptosis and an apparently prodeath autophagy in sensitive cells (68). However, resistant cells that are resensitized to antiestrogens by inhibition of BCL2 and/or BCL-W do not die through apoptosis but through an autophagy-associated necrosis (57). When BECN1 is then also inhibited, necrosis (PCD-3) is no longer a dominant cell-death mechanism and the cells recover the ability to die through apoptosis. Thus, the cell-fate decisions associated with regulation of BCL2 family members and BECN1 are differentially regulated depending on the cellular contexts in endocrine-sensitive and -resistant breast cancer cells (57). Death receptor-5 (DR5, TNFRSF10B), a major component of the extrinsic apoptosis pathway, is regulated by CHOP [GADD153, DNA damage-inducible transcript (DDIT)] that is activated by both PERK and ATF6 (69). CHOP also regulates BCL2 expression (70), which likely concurrently affects its role in both apoptosis and autophagy.

Although p53 is strongly implicated in the regulation of apoptosis, its role in UPR-associated signaling is unclear. Limited evidence suggests a dual role for p53 with respect to autophagy. Genomic stress can induce an apparent p53-dependent autophagy and stimulate the transcription of autophagy-related genes. Conversely, deletion or inhibition of p53 can also activate autophagy (71). Currently, a definitive mechanistic link among antiestrogens, autophagy, and p53 remains to be established. Studies exploring the role of antiestrogen therapies and autophagy using both MCF7 (p53 wild type) or T47D (p53 null) breast cancer cell lines show a broadly similar activation of autophagy in response to endocrine therapy. For example, inhibition of autophagy using either RNA interference or chemical inhibitors potentiates antiestrogen-mediated cell death (72), suggesting that p53 may not play a central role in mediating antiestrogen-induced autophagy.

Changes in intracellular Ca^{2+} and activity of the Ca^{2+}-binding protein calmodulin are implicated in responsiveness to antiestrogens (73). Increased cytosolic Ca^{2+} induces a BECN1/ATG7-dependent, BCL2-sensitive autophagy by activating calcium/calmodulin-dependent protein kinase II beta (CAMK2B) and AMPK, which then inhibits mTOR (74). An AMPK-independent pathway involving the protein phosphatase WIP1 (PPM1D) and LC3 is also implicated in Ca^{2+}-mediated autophagy (75). JNK phosphorylation of BCL2 and its consequent release from BECN1 allow BCL2 to bind and inhibit the function of inositol 1,4,5,-triphosphate receptor [IP3R, ITPR1 (76, 77)]. IP3R controls the release of Ca^{2+} from the ER into the cytosol, and a decrease in Ca^{2+} can delay or reduce apoptosis. This activity may be unrelated to its role in autophagy (78); rather, the concurrent release of BECN1 is likely to be the regulator of autophagy. Cleavage of ATG5 by the calcium-dependent, nonlysosomal cysteine protease calpain can also cause a transition from autophagy to apoptosis (79). Although the precise role of Ca^{2+}-mediated signaling may be complex and cell-context–dependent, these observations provide further evidence of how components common

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to UPR, apoptosis, and autophagy may coordinately affect their relative activation.

Coordination of cellular metabolism and cell fate

An appropriately activated UPR can eliminate ER stress, restore correct protein folding, and allow a cell to function normally (the prosurvival function). UPR activation of ERAD may support the recycling of material recovered from the degradation of misfolded proteins, which could also allow cells to survive when extracellular nutrient sources are limited. A link between cell fate and UPR is consistent with the use of ERAD(II) to eliminate insoluble misfolded proteins through an autophagic process. The eventual dissolution of autolysosomes during autophagy releases the degraded or partially degraded macromolecules from damaged or unnecessary organelles and cytosolic contents for subsequent reuse. Autophagy can be initiated by several stressors, including the persistent nutrient deprivation that may arise from inadequate vascularization and/or loss of stimulation by growth factors [e.g., insulin-like growth factor (IGF)]. However, precisely how nutrient deprivation is sensed is not entirely clear. mTOR can integrate signaling from insulin, growth factors such as IGF-I and IGF-II, and amino acids (80, 81). Nutrient/energy deprivation-regulated signaling may also include activation of AMPK by means of an increased AMP:ATP ratio (ATP depletion), or induction of REDD by HIF1 in response to hypoxia/oxidative stress, which can lead to inactivation of the TORC1 complex and release its repression of autophagy (82, 83).

p53 is altered in 20% to 40% of all breast carcinomas (84) and was recently implicated in the regulation of metabolism. For example, decreased oxygen consumption and increased glycolytic activity occur in $p53^{-/-}$ mutant mice, with no overall change in total ATP production. Altered metabolism is linked to p53-mediated transcriptional regulated targets, such as mitochondrial cytochrome oxidase c (COX)-complex, with an observed increase in lactate accumulation (85). Low pH can stimulate AMPK and p53 expression, resulting in a high glycolytic flux and inhibiting apoptosis through increased expression of BCL2 and p53 (TP53)-induced glycolysis and apoptosis regulator [TIGAR (86)]. p53-induced TIGAR expression protects cells against oxidative stress and regulates glycolysis (87). Given the high frequency of p53 mutations observed in breast cancer, the role of p53 in the possible coordination of UPR signaling, antiestrogen resistance, and metabolism clearly requires further study.

In cancer cells, insufficient glucose or other energy substrates may create low intracellular ATP concentrations. Moreover, as intracellular glucose levels fall, members of the glucose-regulated protein family are activated (88). This family includes HSPA5, and low glucose can result in the release of HSPA5 from the UPR sensor proteins and activation of the UPR. Thus, activation of glucose-regulated proteins provides another general means of sensing nutrient insufficiency and inducing a UPR-regulated autophagy. Whatever the upstream activation, once autophagy is initiated, it can enable metabolite recycling and contribute to the restoration of metabolic homeostasis.

Further study is needed to determine precisely how the contents released from autolysosomes feed into a cancer cell's energy metabolism, which generally has a high glycolytic demand due to the Warburg Effect, or into its intermediate metabolism to maintain or replace basic cellular components. Intermediate metabolism may be largely intact, and the reuse of amino acids, peptides, carbohydrates, and small fatty acids may ultimately feed into the tricarboxylic acid (TCA) cycle in adequately functional mitochondria. Larger fatty acids are probably metabolized in peroxisomes, as would also be the case in most cells. PPARs may play a major role during metabolic stress by ensuring adequate turnover of peroxisomes to manage the greater metabolic requirement for release of the energy stored in the longer-chain fatty acids that are provided by autophagy (Fig. 2D). This is also likely to be a dominant role for PPARs during stress. Similarly, the primary roles of insulin and the IGFs may be to affect autophagy and basal survival metabolism, including regulation of glucose metabolism. However, they may only be able to increase proliferation if cellular metabolism permits. Whether growth factors or other mitogens activate proliferation is probably a secondary concern for a cancer cell, because the ability to survive, even in an essentially dormant (nonreplicative) state, is likely preferable to death. Thus, it is not surprising that growth factors, hormones, and other mitogenic signals involve a coordinated regulation of metabolism, cell survival, and cell cycling. We propose that this regulation is often hierarchical, or at least appears to be so. Because both survival and PCD mechanisms are energy-dependent, and the choice to live or die may be determined by metabolic status, the hierarchical importance for cellular decision-making may be ordered as follows: signaling to regulate metabolism (highest priority) → survival → proliferation (lowest priority). As such, by focusing on efforts to therapeutically target replication, investigators may miss the potential of targeting metabolism, provided that can be done in a manner that does not also adversely affect non-cancer cells and induce excessive toxicity.

UPR and the tumor microenvironment

In addition to the role played by UPR-mediated control of autophagy and apoptosis in regulating tumor cell fate, as clearly highlighted in this review, recent studies suggest that UPR signaling also affects interactions within the tumor microenvironment. A transgene-induced mammary tumor model in HSPA5 heterozygous knockout mice exhibited decreased angiogenesis and tumor microvessel density (89). In a syngeneic breast tumor model, wild-type tumor cells implanted into a HSPA5 heterozygous mouse showed decreased angiogenesis in early- but not late-phase tumor growth, and the number of metastatic lesions was also reduced in the HSPA5 heterozygous animals (90). Knockdown of HSPA5 in endothelial cells decreased their proliferation, survival, and migration, implicating UPR in angiogenesis within the tumor microenvironment (90). In contrast, increased expression of HSPA5, GRP94 (HSP90B1), and protein disulfide isomerase (PDI) was detected in the

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circulating progenitor/cancer stem cells of patients with breast cancer (91). Because UPR signaling may be important in both tumor cells and other cells in the tumor microenvironment, the UPR coordination of cell fate proposed in this review may be broadly applicable to many different cell types.

Conclusions and Future Prospects

Signaling initiated from within the UPR actively participates in autophagy and both intrinsic and extrinsic apoptosis pathways. The latter is logical because ER stress can result from internal or external stressors. Inappropriate activation of the UPR, whether the effect on cell fate is prodeath or prosurvival, can be problematic. Failure to eliminate stressed cells, particularly in cells with damaged DNA, could lead to cancer. UPR activation leading to a prosurvival outcome in preexisting cancer cells would clearly be detrimental to the host. Activation of the UPR may be more likely for cancers that arise from normal cells with a significant secretory function, where UPR activation may be a common occurrence. Cancers of the breast, prostate, immune system, and pancreas are among the most common cancers and are strong candidates to exhibit a central role for UPR activation as a cell-survival mechanism. Nonetheless, this general function is conserved in evolution and may be active in most cancers. Cancer cells generally experience multiple cellular stressors associated with the UPR, including nutrient deprivation from inadequate vascularization (92) or exposure to endogenous and/or treatment-induced oxidative stress (93, 94).

Signaling that is initiated within the UPR, or is external to the UPR but uses some of its signaling components, can influence the initiation of both apoptosis and autophagy and contribute to the cell-fate decision process. Integration of this signaling is critical if the cell is to use UPR and autophagy first to determine whether it should or can survive. It would be pointless to initiate a stress-response pathway to resolve the stress if an irreversible cell death signal were concurrently activated. Thus, cell signaling appears to be wired so that the same molecules, such as BCL2 family members, can concurrently repress one function (such as prodeath) while activating an opposing activity (prosurvival). For example, the association of IRE1α with the proapoptotic BAK and BAX affects the UPR (67), suggesting one mechanism by which apoptosis could be inhibited while the cell tries to use a UPR-mediated autophagy to recover.

Given that UPR and autophagy have integrated and perhaps interdependent functions, it is not surprising that both can be associated with prodeath and prosurvival outcomes. How these interactions differ between cancer and normal phenotypes, or between drug-sensitive and drug-resistant phenotypes, is an area for research. Moreover, although we chose to use the widely described PCD2 for autophagy in the context of cell death, in a recent study of autophagic flux in response to chemically induced stress, Shen and colleagues (95) suggested that the process we usually think of as autophagic cell death may actually be a very rare cell-fate outcome. This intriguing observation requires small topologic features of the network that controls endocrine responsiveness have been identified from within gene expression microarray data additional study, but it may also require a revision in how we think of autophagy as a mechanism for executing cell death (95). Regardless of whether autophagic cell death occurs, the plasticity of the cell-fate decision and the importance of cellular context are already evident. Plasticity and context can each exist within one integrated signaling network, each being explained by the presence of an adaptive network topology. For example, the nodes of the signaling network that determines cell fate may be largely maintained even though the frequency, strength, and direction of their interactions (edges) are changed (58). Such topologic changes could be further modified by perturbations in the set-points required to activate irreversible decisions (96). Also, the relative importance of a node or edge could be modified by a change in sequence (e.g., mutation or splicing), transcription, translation, post-translational modification, and/or subcellular localization.

Cellular signaling occurs in the context of interactive networks (58, 97), and a considerable degree of integration and communication occurs among the signals associated with the UPR, autophagy, and apoptosis. It is unlikely that we will be able to represent, understand, and explore such complex processes by attempting to capture information in static wiring diagrams such as we have used here to illustrate some signaling transduction. Such diagrams are necessarily simple, and many potential nodes are already available for inclusion in a model that might explain cell-fate decisions, such as those activated in response to endocrine therapies in breast cancer (98, 99). Instead, we may need to use a systems approach involving both computational and mathematical modeling to construct hypotheses that will better identify the most important and informative experiments, and ultimately enable the testing of predictions about how the system responds to stress and makes irreversible cell-fate decisions (58).

Despite the many challenges of working in high-dimensional data spaces (97), small topologic features of the network that controls endocrine responsiveness have been identified from within gene expression microarray data (44, 100). A framework for mathematical modeling of cell-fate decision-making in the context of responsiveness to endocrine therapies in breast cancer was recently proposed (101). The model incorporates modules for the cell cycle, apoptosis, autophagy, and the UPR. Models for some individual modules, including the UPR, have also been proposed (27). However, the current models are generally high level, and there is a notable paucity of data we can use to define the parameters and construct informative and sufficiently robust mathematical models for any of these critical functions and their regulatory components. Finally, it is evident that studies involving therapy responsiveness and cell-fate decisions require careful consideration of the integrated role of UPR, autophagy, apoptosis, and necrosis. It also seems likely that novel therapeutic targets reside within this network (102). It remains to be seen how these opportunities can be identified and used to good effect in the attempt to eradicate cancer of the breast and other cancers.

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No potential conflicts of interest were disclosed.

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EXPERT REVIEWS

Autophagy and endocrine resistance in breast cancer

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The American Cancer Society estimates that over 200,000 new breast cancer cases are diagnosed annually in the USA alone. Of these cases, the majority are invasive breast cancers and almost 70% are estrogen receptor-α positive. Therapies targeting the estrogen receptor-α are widely applied and include selective estrogen receptor modulators such as tamoxifen, a selective estrogen receptor downregulator such as Fulvestrant (Faslodex; FAS, ICI 182,780), or one of the third-generation aromatase inhibitors including letrozole or anastrozole. While these treatments reduce breast cancer mortality, many estrogen receptor-α-positive tumors eventually recur, highlighting the clinical significance of endocrine therapy resistance. The signaling leading to endocrine therapy resistance is poorly understood; however, preclinical studies have established an important role for autophagy in the acquired resistance phenotype. Autophagy is a cellular degradation process initiated in response to stress or nutrient deprivation, which attempts to restore metabolic homeostasis through the catabolic lysis of aggregated proteins, unfolded/misfolded proteins or damaged subcellular organelles. The duality of autophagy, which can be either pro-survival or pro-death, is well known. However, in the context of endocrine therapy resistance in breast cancer, the inhibition of autophagy can potentiate resensitization of previously antiestrogen resistant breast cancer cells. In this article, we discuss the complex and occasionally contradictory roles of autophagy in cancer and in resistance to endocrine therapies in breast cancer.

KEYWORDS: 3-methyladenine • antiestrogen resistance • aromatase inhibitor • autophagy • bafilomycin A1 • breast cancer • endoplasmic reticulum stress • fulvestrant • hydroxychloroquine • tamoxifen • unfolded protein response

Endocrine therapy resistance in estrogen receptor-α-positive (ER+) breast cancer, whether acquired or *de novo*, remains an important clinical problem. While adjuvant endocrine therapy reduces breast cancer mortality, many ER+ tumors will eventually recur. Mechanisms of antiestrogen resistance are still poorly understood; however, preclinical studies suggest that several druggable targets offer the potential to restore endocrine therapy sensitivity, such as key components of prosurvival autophagy signaling. Autophagy, or 'self-eating', is a mechanism by which a cell digests its own subcellular organelles or unfolded/misfolded/aggregated proteins. Under normal conditions, this provides a quality-control mechanism, removing damaged organelles and proteins. In response to a stressor, this autophagic digestion recovers energy in an attempt to maintain/restore metabolic homeostasis. Targeting autophagy through chemical inhibitors, such as hydroxychloroquine (HCQ) or 3-methyladenine (3-MA), or by RNAi targeting of *beclin-1* can restore antiestrogen sensitivity in some resistant breast cancer cells. Several clinical trials have been initiated to investigate the role of autophagy in different cancer types, including metastatic breast cancer. One clinical trial of particular interest in this regard is the Preventing Invasive Breast Neoplasia with Chloroquine (PINC) study, which involves the inhibition of autophagy while concurrently treating with tamoxifen in patients with ductal carcinoma *in situ* of the breast.

The American Cancer Society (ACS) estimates that over 200,000 new cases of breast cancer are diagnosed in the USA each year [1]. Breast cancer remains the second highest killer of all cancers in women, second only to lung and bronchial cancer, with more than 40,000 reported deaths in women in the USA last year [1,2]. Over 1.15 million new cases of breast cancer are estimated to have been diagnosed worldwide last year, resulting in over 411,000 deaths in women. Breast cancer is the leading cause of cancer mortality in women worldwide [3]. Therapies targeting the ER are widely applied and include selective estrogen receptor modulators such as tamoxifen (TAM), a selective estrogen receptor

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downregulator such as Faslodex (FAS; fulvestrant [FAS], ICI 182,780), or one of the third-generation aromatase inhibitors (AIs) including letrozole or anastrozole. Antiestrogens are less toxic than cytotoxic chemotherapy and TAM has represented the 'gold standard' in first-line endocrine therapy for over 30 years [4]. More recently, AIs have begun to replace TAM as the first-line endocrine therapy of choice for ER+ postmenopausal breast cancer [5]. FAS, an antiestrogen drug lacking the agonist estrogenic affects of TAM, downregulates the ER through enhanced ubiquitin-mediated degradation of the receptor and has a different modality of action when compared with TAM [6,7]. In some patients, FAS is as effective as an AI [8]. While clinical studies demonstrate that adjuvant endocrine therapy reduces mortality, many ER+ breast tumors that initially respond to therapy develop acquired resistance [9–11]. For the most part, advanced ER+ breast cancer remains an incurable disease, highlighting the importance of understanding endocrine therapy resistance.

Two different types of antiestrogen resistance are generally described, *de novo* or intrinsic resistance and acquired resistance. A primary mechanism of *de novo* resistance to antiestrogen therapy is the lack of detectable ER expression [12,13]. Acquired resistance appears to occur through many different mechanisms, several of which involve changes in the ER including mutations, altered patterns of phosphorylation by growth factors and their downstream kinases, and altered expression of ER coregulators [13]. Much of our current understanding of antiestrogen resistance is based on studies focused on TAM resistance in experimental models of breast cancer. While these endocrine resistance studies have implicated many causative genes (reviewed in [13,14]), more recent studies associate autophagy and cell stress responses with endocrine resistance and thus open up a new area of research in this field (see recent reviews [15,16]).

Autophagy

Autophagy (macroautophagy) is a conserved evolutionary process that can enable cells to maintain homeostasis in unfavorable environmental conditions. An autophagic 'self-eating' allows the cell to recover energy from damaged or unnecessary subcellular components. However, if the insult is too severe and autophagy persists at a high level, it becomes pro-death; an autophagic cell death is often referred to as programmed cell death-2 (apoptosis is programmed cell death-1). Basal levels of autophagy help clear injured organelles or long-lived proteins; hypoxia, nutrient or growth factor deprivation, accumulation of misfolded or unfolded proteins in the endoplasmic reticulum or infection can each increase the extent of autophagy (reviewed in [17]).

The process of autophagy involves the segregation of cytoplasm and intracellular organelles in double membrane-bound structures called autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes, facilitating the degradation of the sequestered cellular material by lysosomal hydrolases. Under starvation conditions, degraded organelles or proteins are recycled and converted into metabolic intermediates that can be used to fuel the cell. Under hypoxia, autophagy removes reactive oxygen species-generating mitochondria, thereby protecting the cell [18].

The autophagy-related family of proteins (Atg) comprise the distinct molecular machinery necessary for the induction and formation of autophagosomes, autophagosome-vesicle fusion, lysis and release of degraded molecules back into the cytosol (reviewed in [19]). Table 1 summarizes the primary autophagy-related genes and their effects on the autophagy pathway. The process of autophagy is best defined in yeast. Critical to the initiation of autophagy is the activation of Atg1 (mammalian homolog: Unc-51-like kinase [ULK]-1 and -2), which is negatively regulated by the serine/threonine protein kinase target of rapamycin (TOR) [20,21]. Under low-nutrient conditions where TOR is repressed, the kinase activity of Atg1 enables binding of Atg1 to Atg13 and Atg17 (mammalian homolog: focal adhesion kinase family-interacting protein of 200 kD; FIP200 and RB1CC1), thereby creating a scaffold for recruiting other Atg proteins [22]. Unlike yeast, mammalian cells can form stable ULK-Atg13-FIP200 complexes regardless of nutrient conditions.

In mammalian cells, autophagosome degradation is driven by p62/sequestosome-1 (SQSTM1), which binds directly to ubiquitinated proteins and microtubule-associated protein-1 (MAP1) light chain-3 (LC3), linking the ubiquitinated proteins to the autophagic machinery [23–25]. Formation of the autophagosome double membrane occurs *de novo* and not from either pre-existing organelles or by the annealing of single membranes, and requires the actions of vacuolar protein sorting 34 (Vps34), p150, Atg4 and beclin-1 (BECN1) [26–28]. Beclin-1 activity is regulated by B-cell lymphomia/leukemia-2 (Bcl-2) and is discussed more thoroughly later. LC3 determines autophagosome size and membrane curvature [19]; the Atg12/Atg5/Atg16 complex and the LC3-phosphatidylethanolamine (LC3-PE or LC3-II) complex participate in elongation of the autophagosome membrane. Atg9 is the only integral membrane protein identified in autophagosome formation, where it may function as a carrier of membrane materials. Atg9 is dependent on ULK1 and Atg13 for transportation from the trans-Golgi network to late endosomes [29–33]. The cysteine protease Atg4 cleaves pro-LC3 to expose a C-terminal glycine residue, enabling Atg12-Atg5 to conjugate LC3 to PE, via an amide bond; LC3-PE levels are often used as a measure of autophagy induction (reviewed in [34]). The early autophagosome fuses with a lysosome to form the late autolysosome. This fusion process is dependent upon the lysosomal membrane protein-2 (LAMP-2) and the small GTPase Rab7 [35,36]. After fusion of the lysosome, the resulting autolysosome degrades its protein/organelle load and inner membrane. In mammalian autophagy, degradation occurs through the actions of cathepsins B, D and L [17,37]. The resulting products of the catalytic degradation process are then transported to the cytosol and recycled. The process of autophagy described above is shown in Figure 1.

Bcl-2 & the regulation of autophagy in breast cancer

The Bcl-2 family contains two distinct functional groups, the antiapoptotic group that includes Bcl-2 and Bcl-XL, and the proapoptotic group including the Bax and Bak proteins. Bcl-2 is an antiapoptotic protein that exhibits oncogenic potential through its ability to regulate the intrinsic apoptotic pathway. The molecular

Table 1. Selected autophagy-related genes.

Autophagy gene	HUGO gene symbol	Effect on autophagy
Atg3	*ATG3*	E2-like enzyme facilitates lipidation of LC3
Atg4A, *-B*, *-C*, *-D*	*ATG4A*, *ATG4B*, *ATG4C* and *ATG4D*	Cleaves pro-LC3 to form LC3
Atg5	*ATG5*	Forms a complex with Atg12-Atg16, resulting in lipidation of LC3
Atg7	*ATG7*	E1-like enzyme activates Atg12
Atg9A, *-B*	*ATG9A* and *-B*	Phagophore membrane expansion
Atg10	*ATG10*	E2-like enzyme facilitates the formation of Atg5-Atg12-Atg16 complex
Atg12	*ATG12*	Forms complex with Atg5-Atg16, resulting in lipidation of LC3
Atg13	*ATG13*	Part of the initiation complex with ULK1, Atg101 and FIP200
Atg16L1, *-L2*	*ATG16L1* and *-2*	Forms a complex with Atg5-Atg12, resulting in lipidation of LC3
Beclin-1	*BECN1*	Part of the initiation complex with Vps34
Atg101	*C12orf44*	Part of the initiation complex with ULK1, FIP200 and Atg13
Cathepsin B	*CTSB*	Lysosome enzyme
LAMP1, *-2*, *-3*	*LAMP1*, *-2* and *-3*	Lysosome autophagosome fusion
LC3 (A, B or C)	*MAP1LC3A*, *-B* and *-C*	Phagophore membrane curvature and expansion
MTOR	*MTOR*	Inhibits ULK1
PIK3C3 (Vps34)	*PIK3C3*	Part of the initiation complex with beclin-1
FIP200	*RB1CC1*	Part of the initiation complex with ULK1, Atg101 and Atg13
p62	*SQSTM1*	Cargo recognition
Rab7	*RAB7A*	Lysosome autophagosome fusion
ULK1	*ULK1*	Part of the initiation complex with Atg101, Atg13 and FIP200

HUGO: Human Genome Organisation; LAMP: Lysosomal membrane protein; LC: Light chain; ULK: Unc-51-like kinase.

activity of Bcl-2 involves binding to mitochondrial Bax, thereby preventing Bax activation, mitochondrial outer-membrane permeabilization and apoptosis. Bcl-2 is overexpressed in over 60% of breast tumors; overexpression of Bcl-2 correlates with chemotherapeutic and radiation resistance [38,39]. Moreover, a recent clinicopathological investigation that measured both beclin-1 and Bcl-2 in breast cancer tissue indicated that beclin-1 is inversely correlated with Bcl-2 expression. Increased Bcl-2 expression is associated with the estrogen receptor, increased histological grade and distant metastases [40]. These data highlight the role of Bcl-2 in breast cancer and resistance.

Beclin-1 was originally identified as a Bcl-2-interacting protein [41], the Bcl-2 family being a group of proteins containing Bcl-2 homology domains. Beclin-1 binds to Bcl-2, Bcl-W, Bcl-X_L and Mcl-1, which results in the inhibition of autophagy [41–43]. Table 2 summarizes the Bcl-2 family members' effects on apoptosis and autophagy. In several cell types, binding of Bcl-2 to beclin-1 inhibits the binding and activation of Vps34, decreasing Vps34-mediated PI3K activation and subsequently inhibiting autophagy. In cases of nutrient starvation, or when cells are treated with Bcl-2 inhibitors that reduce Bcl-2 protein levels, Bcl-2 and beclin-1 dissociate and autophagy is stimulated [41–43]. Conversely, proapoptotic Bcl-2 family members, such as Bad, Bik, BNIP3L, Noxa, Puma and Bim_{EL}, may induce autophagy by competitively binding to Bcl-2 family members and disrupting the interaction between beclin-1 and Bcl-2; thereby freeing beclin-1 [44–46]. A recent study into the role of Mcl-1 in autophagy reported that, in response to glucose deprivation and hypoxia, Mcl-1 is rapidly degraded and autophagy becomes activated. Furthermore, Mcl-1 overexpression prevents LC3-positive punctate formation, indicating a key regulatory role of Mcl-1 in autophagy [47].

Several Bcl-2 inhibitors are currently undergoing clinical trials. While the use of Bcl-2 inhibitors are predominately focused on leukemias and lymphomas, a potential role for these inhibitors in breast cancer is now evident [48]. For example, preclinical studies investigating the role of Bcl-2 in MCF-7 breast cancer cells show that silencing Bcl-2 by siRNA increases autophagy and cell death, highlighting the possible use of Bcl-2 inhibitors as a therapeutic strategy in breast cancer [49]. Gossypol, a BH3 mimetic isolated from cotton seeds, induces beclin-1-dependent and -independent autophagy, resulting in cytoprotection and survival of MCF-7 breast cancer cells [50]. These studies likely reflect an important role for Bcl-2 family members in the regulation of autophagy in breast cancer.

Unfolded protein response & the regulation of autophagy in breast cancer

The unfolded protein response (UPR) pathway is activated in response to the accumulation of aggregated unfolded/misfolded proteins within the endoplasmic reticulum (EnR) lumen.

Table 2. Role of Bcl-2 family members on apoptosis and autophagy.

Bcl-2 family member	HUGO gene symbol	Effect on apoptosis	Effect on autophagy
Bcl-2	*BCL2*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Bcl-w	*BCL2L2*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Bcl-X_L	*BCL2L1*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Mcl-1	*MCL1*	Antiapoptotic	Inhibits autophagy (to a lesser extent than Bcl-2, Bcl-w and Bcl-X_L) by binding to beclin-1
Bad	*BAD*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
t-Bid	*BID*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bim_{EL}	*BCL2L11*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Noxa	*PMAIP1*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Puma	*BBC3*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
BNIP3L	*BNIP3L*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bik	*BIK*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bax	*BAX*	Proapoptotic	No effect
Bak	*BAK1*	Proapoptotic	No effect

HUGO: Human Genome Organisation.

In response to this accumulation, the EnR protein chaperone glucose-regulated protein 78 (GRP78; BiP; HSPA5) is released from each of inositol requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase, enabling their respective activation. IRE1 dimerizes and becomes autophosphorylated, resulting in its activation and ability to perform the unconventional (cytosolic) splicing of the X-box binding protein-1 (XBP1) mRNA. XBP1 splicing creates the transcriptionally-active XBP1-S form [16,51], which can confer estrogen independence and antiestrogen resistance upon estrogen-dependent breast cancer cells [16,52] and is now known to be associated with a poor response to TAM [53].

Unfolded protein response stimulation promotes the activation of autophagy through different mechanisms. EnR stress results in phosphorylation of eIF2α by PKR-like endoplasmic reticulum kinase. Activated eIF2α increases ATF4 expression, which then increases the transcription of Atg12 and can thereby promote autophagy [54]. IRE1 activation also leads to the phosphorylation of c-Jun-terminal kinase, resulting in the phosphorylation of Bcl-2 at the T69, S70 and S87 residues in the unstructured loop of Bcl-2 [55]. Phosphorylation of Bcl-2 can cause dissociation of the Bcl-2/beclin-1 complex and thus may activate autophagy. Figure 2 illustrates the interaction between UPR signaling and autophagy. Calcium released from the EnR following stress also promotes autophagy by activating beclin-1 [17]. Therefore, the stimulation of UPR by mechanisms such as nutrient starvation, hypoxia or therapeutic drugs may result in the subsequent activation of a prosurvival autophagy.

Dichotomy of autophagy in cancers

In cancer, autophagy can serve as either a 'tumor suppressor' or as a 'tumor promoter'. Allelic loss of vital autophagy components, such as beclin-1, is often found in breast, ovarian and prostate cancers [56–59]. Moreover, inhibition of beclin-1 or deletion of Atg5 in immortalized epithelial kidney cells or breast cancer cell lines is associated with increased proliferation and tumorigenicity [60,61]. Genetically altered heterozygous beclin-1 knockout mice exhibit an increased incidence of hepatocellular carcinoma, lung adenocarcinoma and mammary hyperplasia [62]. Increased susceptibility to hepatitis B-induced hepatocellular carcinoma, when compared with their wild-type counterparts [63,64], is also reported. Brain tumors often have reduced beclin-1 compared with the normal surrounding tissue and reduced beclin-1 inversely correlates with malignancy [65]. Vps34 expression is also dysregulated in cancers. Vps34 overexpression in colon cancer cell lines reduced tumorigenicity, while heterozygous deletion of the Vps34 gene *uvrag* is often observed in colon tumors [66]. Knockdown of Atg4, the protease that cleaves LC3, increases the severity of chemically-induced fibrosarcomas in mice [67]. These data suggest that disruption of the autophagic process is a key event in tumorgenesis.

While direct modulation of the components of autophagy is observed in different cancers, mutations indirectly affecting autophagy are also reported. *PI3K* mutations are found in over 20% of breast cancers and 30% of colorectal cancers. These mutations may indirectly influence autophagy through the stimulation of mTOR, which would prevent ULK activity and inhibit autophagy [62]. Another possible autophagy-regulating event, *p53* mutational inactivation, is observed in over 50% of all tumors [68]. Inactivating *p53* mutations, mutation in the *p53* activating kinases, overexpression of MDM2 that degrades p53 and loss of function of $p14^{ARF}$, are each documented in various cancers and these result in a loss/reduction in p53 activity (reviewed in [45]). Nuclear p53 can affect autophagy through transactivation of death-associated protein kinase-1 (DAPK-1) and the lysosomal protein damage-regulated autophagy modulator [69]. DAPK-1 is commonly dysregulated in

human tumors and has both proapoptotic and proautophagy activities. For example, DAPK-1 phosphorylates myosin light chain, promoting membrane blebbing and apoptosis [70]. DAPK-1 is also implicated in autophagy through its ability to bind MAPB1, which interacts with LC3 to inhibit autophagy [70,71]. Transactivation of DRAM by p53 can activate autophagy and is necessary for the execution of a DNA damage-induced p53-mediated cell death. Furthermore, p53 inhibits mTOR and can thus activate autophagy.

While the transcriptional activation of *p53* promotes autophagy, cytosolic p53 can inhibit autophagy [72]. Preclinical studies show an increase in autophagy with an increased formation of LC3-containing autophagic vacuoles when p53 is knocked down, knocked out or otherwise inhibited [34,73]. Moreover, inhibition of nuclear transporters, resulting in the accumulation of p53 in the nucleus, prevents the inhibitory actions of p53 on autophagy. While the influence of p53 on autophagy is evident, the overall effect of p53 on the regulation of autophagy remains controversial. Function of the upstream activator of p53, $p14^{ARF}$, is also lost in many cancers and the mitochondrial form of $p14^{ARF}$ is a potent stimulator of autophagy [74].

Defects in autophagy may also promote tumorgenesis. Impaired autophagy hinders the ability of a cell to survive stressful environmental conditions and can result in increased cell death [59,60]. While this may seem anti-tumorigenic at first, chronic cell death leads to a prolonged inflammatory response that can be oncogenic. Cancer-related inflammation is often considered the seventh hallmark of cancer [75]. For example, chronic cell death in the liver stimulates inflammation, increases organ damage and raises the risk of developing hepatocellular carcinoma. Necrotic cell death leads to the release of cellular debris, activating various cell-surface receptors on neighboring cells that can stimulate survival pathways and enhance cell growth [75,76]. Moreover, autophagy also limits genotoxic damage by reducing the formation of reactive oxygen species (ROS) and clearing damaged mitochondria [75,77]. When autophagy is impaired, damaged mitochondria remain in the cell, increasing ROS production and the associated protein, organelle and DNA damage. Oxidative damage from the accumulation of ROS may promote tumorgenesis, thereby supporting inadequate autophagy as a contributor to oncogenesis (reviewed in [76]).

While autophagy is implicated as a tumor supressor in early tumorgenesis, a growing body of evidence implicates autophagy as a tumor promoter in late stage cancers. The ability of the autophagic process to provide cellular resilience to stressors such as glucose deprivation and hypoxia, two common stresses experienced by tumors, enables long-term cell survival [45,57,60,78]. Autophagy allows cancer cells to 'eat themselves', progressively reducing in size, to conserve and provide nutrients for survival functions. Since some of these cancer cells retain the ability of self-renewal, they can return to their original size and proliferate given proper conditions. Thus, some cancer cells with intact autophagy may exhibit cellular dormancy (reviewed in [59]).

In summary, the oncogenic activity of autophagy stimulation may be stage dependent. Autophagy appears to a tumor suppressor in early tumorigenesis, perhaps because the cells cannot easily adapt to the increased elimination of subcellular organelles as an energy source. During progression, those cells that survive will likely have been able to adapt their physiology such that they can survive with the increased basal level of autophagy, which may now be providing sufficient energy for survival without exceeding a threshold where cell death becomes inevitable.

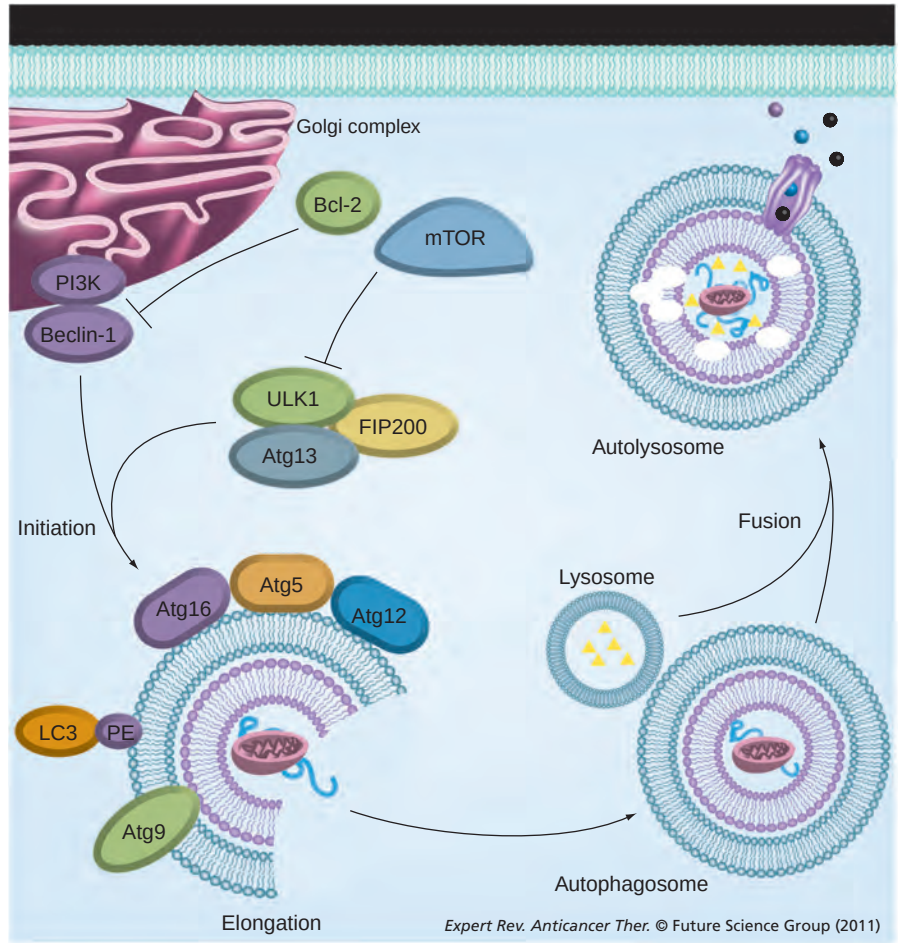

Figure 1. Cellular pathway of autophagy. The PI3K complex mediates the initiation of the phagophore membrane, enveloping labeled cytosolic proteins organelles and fat. mTOR and Bcl-2 can inhibit the initiation of autophagy. The Atg12-Atg5-Atg16 complex, LC3 and the transmembrane Atg9 are recruited to the phagophore and are necessary for elongation of the double membrane. Lysosomes fuse with the autophagosome, creating the autolysosome. The resulting products of the catalytic degradation process are transported to the cytosol and recycled.
LC: Light chain-3.

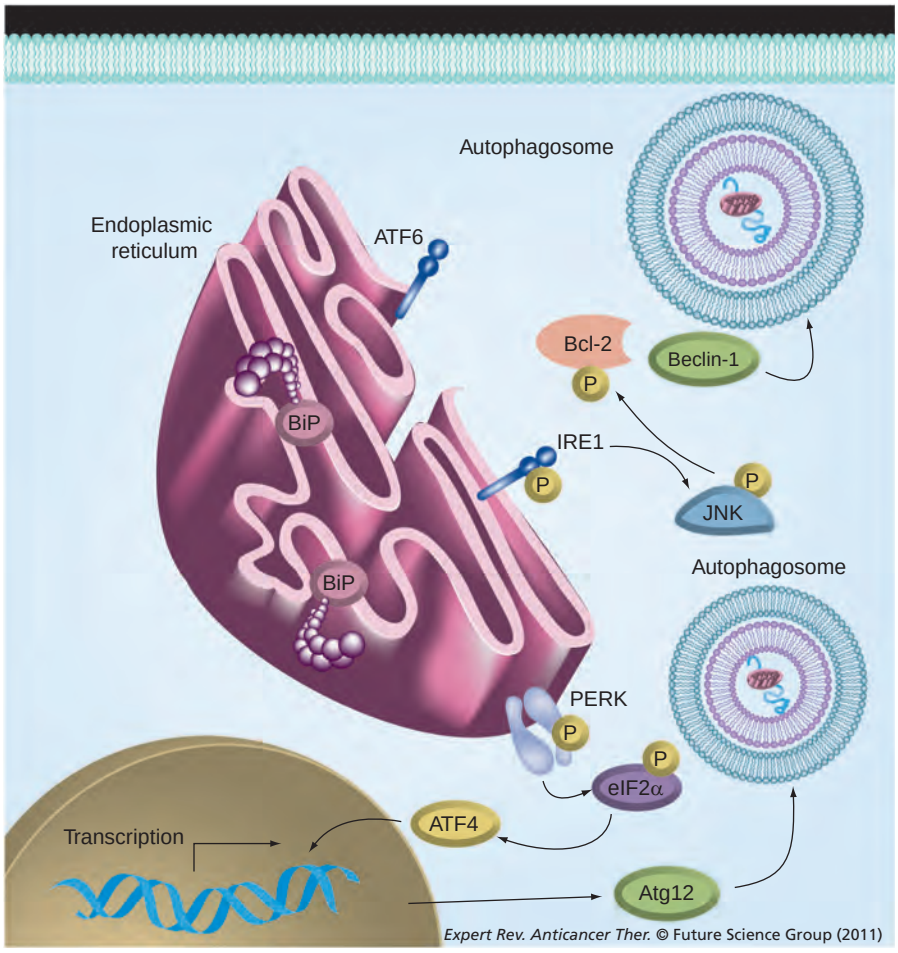

Figure 2. Effect of the unfolded protein response on autophagy. The unfolded protein response can activate autophagy through two distinct mechanisms. PERK activation leads to phosphorylation of eIF2α, resulting in increased ATF4 transcription. ATF4 promotes the transcription of Atg12, resulting in increased autophagy. Another mechanism of unfolded protein response-modulating autophagy is through activation of IRE1. IRE1 activates JNK, leading to the subsequent phosphorylation of Bcl-2. Bcl-2 phosphorylation prevents its binding to beclin-1, thereby promoting autophagy.
ATF: Activating transcription factor; IRE1: Inositol requiring enzyme-1; PERK: PKR-like endoplasmic reticulum kinase.

Autophagy in breast cancer

The role of autophagy in breast cancer, like other types of cancer, is an area of active investigation. Clinicopathological investigation of breast cancer tissue indicated three discernable LC3-positive patterns; diffuse cytoplasmic, cytoplasmic/juxtanuclear and dense round 5 µm 'stone-like' structures [79]. Diffuse cytoplasmic or cytoplasmic/juxtanuclear LC3 staining correlates directly to with estrogen and progesterone receptor expression in breast tissue. Moreover, the 'stone-like' LC3 stained phenotype is associated with high-grade tumors and a less favorable outcome, suggesting the more autophagic a tumor, the more aggressive the tumor [79].

Evidence also supporting the oncogenic activity of autophagy was obtained through 3D morphogenic assays investigating the role of beclin-1 on mammary acini formation. Immortalized mouse mammary epithelial cells with homozygous beclin-1 produce an atypical solid acini structure, owing to the autophagy-competent central acini epithelial cells having an increased capacity to survive anoikis and the hypoxic environment of the central lumen [61]. Conversely, heterozygous deletion of beclin-1 in Immortalized mouse mammary epithelial cells results in normal mammary lumen formation. The defect in autophagy resulting from beclin-1 deletion leads to necrosis of the central epithelial cells, allowing normal mammary lumen formation [78]. Morphogenesis assays capture the physiological context found in normal tissues to model adhesion signaling in acini formation. The ability of cells to form atypical solid acini parallels breast cancer's invasion and metastatic potential *in vivo*; therefore, these data indicate a role of beclin-1 and autophagy in mammary acini development and in cancer progression. A role for beclin-1 in breast tumorigenesis is apparent in the significant variability of beclin-1 expression across different molecular subtypes; higher expression levels of beclin-1 are seen in the HER2-negative luminal-A or luminal-B breast cancers [80,81]. In combination with the mammary acini study, these data indicate that the role of beclin-1 in breast cancer may be more complex than that of just a tumor suppressor, consistent with data from other cancers.

In triple-negative breast cancer (ER negative, PR negative, HER2 negative), the effect of autophagy is just beginning to be elucidated. Recent studies report increased autophagic properties in the mitochondria of the metastatic triple-negative MDA-MB-231 when compared with the less metastatic MDA-MB-468 and noncancerous MCF7–10A cells [82]. However, investigation into the effect of phytochemical therapy and PPARγ ligands in triple-negative breast cancers shows an increase in cell death caused by autophagic activation and necrosis, suggesting there may be a threshold limitation between prosurvival and prodeath autophagy [83,84]. Various studies of the therapeutic response of experimental chemotherapies in breast cancer have implicated a prodeath role for autophagy. A lipid-modified estrogen derivative, developed to treat breast cancer independent of ER status, was shown to induce apoptosis and autophagy in the triple-negative MDA-MB-231 breast cancer cells [85]. Furthermore, this estrogenic compound interferes with mTOR activity, thereby inducing autophagy and promoting cell death. These observations suggest a possible therapeutic strategy for inhibiting triple-negative or ER-negative breast cancer growth through the stimulation of pro-death autophagy [85].

Autophagy in drug resistance

Preclinical studies using chemical inhibitors of autophagy (described in Table 3) or siRNA to knockdown vital autophagy genes demonstrate the role of autophagy in stress and chemotherapeutic sensitization of cancer cells. Most chemical inhibitors of autophagy lack specificity and often have off-target effects. Preclinical studies using these chemicals may benefit from siRNA knockdown of autophagy genes before concluding that the observed effects are due solely to autophagy inhibition. Inhibition of autophagy in glioblastoma, lung cancer, cervical cancer, prostate cancer, leukemia and breast cancer cells resensitized the cells to various therapeutic agents [86]. For example, upregulation of autophagy can protect cancer cells against various therapies including temozolomide, resveratrol, vitamin D3, anthocyanins, radiotherapy and TAM [16,76,86–89]. Treatment with temozolomide in malignant glioma cells stimulates autophagy without activating apoptosis and is associated with resistance to DNA-alkylating agents in some brain cancers [90]. These data suggest an important role of autophagy in promoting cancer therapeutic drug resistance.

Table 3. Commonly used inhibitors of autophagy.

Compound	Target and effect
Hydroxychloroquine or chloroquine	Lysosomal pH, prevents autophagosome–lysosome fusion
3-methyladedine	Class III PI3K inhibition, prevents autophagosom formation
Wormatin	Class III PI3K inhibition, prevents autophagosome formation
LY294002	Class III PI3K inhibition, prevents autophagosome formation
Bafilomycin A1	Vacuolar ATPase inhibition, prevents autophagosome–lysosome fusion

Breast cancer studies have also revealed a role of autophagy in resistance. Autophagy protects MCF-7 breast cancer cells against epirubicin-mediated cell death, and inhibition of autophagy through beclin-1 siRNA restored epirubicin effectiveness [91]. In addition, when treated with camptothecin or etoposide (DNA-damaging therapeutic drugs) autophagy can delay the onset of apoptotic cell death in breast cancer cells, an effect reversed by knockdown of the autophagy-dependent genes *Atg7* and *beclin-1* [44]. Furthermore, treatment of MCF-7 breast cancer cells with bortezomib, a proteasome inhibitor, results in a potent stimulation of autophagy and UPR. The authors speculate that the observed activation of UPR and autophagy is prosurvival, and therefore may explain the poor response to bortezomib in breast cancer patients [92]. HER2-targeted therapies, such as the monoclonal antibody herceptin and EGF receptor tyrosine kinase inhibitors, such as lapatinib, are sensitive to autophagy-mediated resistance [93,94]. Trastuzumab (herceptin) induces LC3-positive punctate formation in SKBr3 cells (HER2-amplified breast cancer cell line). Inhibition of autophagy by 3-MA and LY294002 increases cell death in response to trastuzumab, suggesting autophagy as a cytoprotective response [94]. Moreover, inhibition of autophagy restores EGF receptor-mediated cell death in lapatinib-resistant BT-474 cells (HER2 amplified breast cancer cell line) [93]. These data suggest that targeting autophagy can be sufficient to restore chemotherapeutic drug sensitivity and promote breast cancer cell death.

Studies have also investigated the outcome of autophagy stimulation in antiestrogen therapy. Bursch *et al.* treated MCF-7 estrogen-dependent ER+ breast cancer cells with the antiestrogens TAM and ICI, and found that dying cells showed increased cytosolic autophagosome formation [95]. These authors concluded that autophagy, stimulated by antiestrogens in MCF-7 cells, resulted in active cell death. However, more recently Samaddar *et al.* suggest that this conclusion more likely reflects cells' failed attempts at survival. Samaddar *et al.* demonstrated that in the surviving MCF-7 cellular population (~70%) after antiestrogen treatment, there was an increase in autophagosome formation. This group also hypothesized that whether autophagy promotes survival or cell death may be dependent on the number of autophagosomes in each cell, resulting in a threshold limit. Inhibiting autophagosome formation via 3-methyladenine (3-MA) or beclin-1 siRNA significantly enhanced antiestrogen-induced cell death in MCF-7 cells, further suggesting a prosurvival role of autophagy in antiestrogen therapy [96]. Qadir *et al.* used siRNA with Atg5, beclin-1 and Atg7 to inhibit autophagy in MCF-7, T47D and TAM-resistant MCF7-HER2 cell lines, and reported that concurrent knockdown of autophagy and treatment with TAM resulted in increased mitochondrial-mediated apoptotic cell death and overall reduced cell viability [97]. Moreover, we have shown that inhibition of autophagy through beclin-1 shRNA or 3-MA treatment in the ICI resistant, TAM cross-resistant MCF7/LCC9 breast cancer cells partially restored antiestrogen therapy effectiveness [89].

Increased responsiveness of resistant breast cancer cells to antiestrogen therapy requires concurrent inhibition of both Bcl-2 and beclin-1. Dual inhibition of Bcl-2 by the chemical inhibitor, YC137 and *beclin-1* knockdown increases apoptosis and decreases cell survival in response to antiestrogen therapy [89]. These data illustrate that inhibition of autophagy pathway, coupled with Bcl-2 inactivation, is more detrimental to antiestrogen resistant breast cancer cell survival than the individual inhibition of either pathway alone. Thus, dual targeting of synergistic molecular pathways may be beneficial to resensitizing antiestrogen-resistant breast cancers. Investigating the role of estrogen signaling in breast cancer cells by *beclin-1* has shown that overexpression of *beclin-1* results in decreased growth in response in estrogen, with a decrease in estrogen-regulated genes including *c-myc*, *c-fos* and *egr 1*. These decreases in estrogenic growth by *beclin-1* overexpression may appear anti-tumorigenic but *beclin-1* overexpression in breast cancer cells also leads to a loss of sensitivity to the antiestrogens raloxifene and TAM, further implying a role for autophagy in promoting antiestrogen resistance [98]. Recent preclinical studies

Table 4. Selected ongoing clinical trials inhibiting autophagy in cancer.

Cancer type	Treatment	Study phase	ClinicalTrials.gov identifier [201]
Glioblastoma multiforme	Hydroxychloroquine, radiation, temozolomide	I/II	NCT00486603
Multiple myeloma	Hydroxychloroquine, bortezomib	I/II	NCT00568880
Advanced non-small-cell lung cancer	Hydroxychloroquine, carboplatin, paclitaxel, bevacizumab	I/II	NCT00728845
Breast (metastatic)	Hydroxychloroquine, ixabepilone	I/II	NCT00765765
Colorectal (metastatic)	Hydroxychloroquine, capecitabine, oxaliplatin, bevacizumab	II	NCT01006369
Prostate	Hydroxychloroquine	II	NCT00726596
Renal cell carcinoma	Hydroxychloroquine, surgery	I	NCT01144169
Breast (DCIS)	Chloroquine, tamoxifen	I/II	NCT01023477
Prostate (metastatic)	Hydroxychloroquine, docetaxel	I/II	NCT00786682
Lung	Hydroxychloroquine, gefitinib	I/II	NCT00809237

DCIS: Ductal carcinoma *in situ*.

into possible drug combinations to overcome autophagy-mediated TAM resistance suggest a possible therapeutic benefit of combining histone deacetylase inhibitors or proteasomal inhibitors with antiestrogens [99,100]. Treatment of antiestrogen-resistant T47D and MCF7 ER+ breast cancer cells with bortezomib produces a potent induction of cell death and an inhibition of autophagy as measured by p62 and LC3 accumulation, suggesting the benefit of targeting autophagy in antiestrogen-resistant breast cancer [99]. Taken together, these data clearly support a role for autophagy in the promotion of antiestrogen resistance in breast cancer.

Since the weight of preclinical data indicates that inhibiting autophagy resensitizes some resistant cancer to specific therapies, it is not surprising that clinical trials targeting autophagy have recently been initiated (Table 4). Since it has been used extensively for the treatment of malaria, safety data for the use of HCQ in humans is already accessible. Clinical trials have been initiated using HCQ in combination with gefitinib in lung cancer, with docetaxel in prostate cancer, with temozolomide in glioblastoma multiforme, with ixabepilone in metastatic breast cancer, and with bortezomib in multiple myeloma. Of particular interest in ER+ breast cancer is a study in ductal carcinoma *in situ*, in which patients will receive TAM, chloroquine or a combination of both for 3 months before surgical removal of the tumor. It will be of great interest to see whether inhibition of autophagy in combination with TAM treatment reduces the growth and invasiveness of these breast tumors. The results of the clinical trials listed in Table 4 should hold promising answers to some of the questions pertaining to the role of autophagy in cancer. Moreover, several groups have recently investigated possible small molecular regulators of autophagy through LC3-GFP imaged-based high-throughput screening [101,102]. It is interesting to note that several of these autophagy modulators, either autophagy inducers or autophagy inhibitors, are already US FDA approved for the treatment of various diseases including cardiovascular disorders, schizophrenia and irritable bowel syndrome.

Expert commentary

Targeting autophagy, particularly when it is acting in a survival mode, has significant potential to lead to the development of novel agents and therapeutic regimens. Existing data already suggest that this could be beneficial in combination with both cytotoxic chemotherapy and with endocrine therapy in some cancers. While it is difficult to predict the outcomes of early trials using 'first-generation' inhibitors such as HCQ, the field is ripe for the development of more specific inhibitors or combinations of new inhibitors. Outcomes from the early trials indicated in Table 4 should begin to offer powerful new insights into these exciting opportunities.

Longer term success in targeting autophagy may require the development of a greater understanding of the signaling that both regulates and executes autophagy. While the basic machinery for its execution is defined in normal systems such as yeast, whether this provides an adequate definition of how autophagy signaling is present or altered in different human cancers remains unclear. Perhaps the greatest opportunity will lie in the identification of cancer-specific modifications in the regulatory signaling, rather than in the execution machinery. Such knowledge may best be obtained by the development of useful computational and/or mathematical models of the signaling-control mechanisms [15].

Five-year view

Greater detail on the control signaling of autophagy will likely emerge and provide new insights into how the extent and duration of prosurvival autophagy are regulated to allow cancer cells to survive for prolonged periods in the presence of natural (nutrient deprivation or immunologic suppression of growth leading to dormancy) or imposed (therapeutic intervention leading to resistance) stress. Data from clinical trials will show some evidence for the activity of autophagy inhibitors, although the full value

of this will await a better understanding of the redundancy in the signals controlling autophagy and the development of combination regimens that address this redundancy. Considering the complexity of this mechanism, systems biology-based approaches will generate the most useful insights, and initial computational and/or mathematical models of autophagy regulation and execution will emerge. While the true clinical potential will likely take longer than 5 years to realize, in part owing to the time needed for clinical follow-up and adequate outcome measures, interest and excitement in this field seems certain to rise substantially within the next 5 years.

Financial & competing interests disclosure

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Key issues

- With over 70% of all breast cancer cases being estrogen receptor-α-positive, endocrine therapy remains the primary treatment for these breast cancer patients.
- Many breast tumors that initially respond to antiestrogen treatments eventually develop acquired resistance; preventing and overcoming antiestrogen resistance remain important clinical goals.
- Autophagy, the processes of 'self-eating', can enable cell survival in adverse environmental conditions, including nutrient deprivation and hypoxia.
- Several cancer therapies induce autophagy, such as radiation, temozolomide, cytotoxic drugs, antiestrogens and aromatase inhibitors.
- Inhibitors of autophagy restore antiestrogen sensitivity in endocrine-resistant breast cancer cells growing *in vitro*.
- Clinical trials involving autophagy inhibitors in combination with endocrine or cytotoxic therapies are now being initiated to study the role of autophagy in the survival and progression of cancers.

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Co-Inhibition of BCL-W and BCL2 Restores Antiestrogen Sensitivity through BECN1 and Promotes an Autophagy-Associated Necrosis

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Abstract

BCL2 family members affect cell fate decisions in breast cancer but the role of BCL-W (BCL2L2) is unknown. We now show the integrated roles of the antiapoptotic BCL-W and BCL2 in affecting responsiveness to the antiestrogen ICI 182,780 (ICI; Fulvestrant Faslodex), using both molecular (siRNA; shRNA) and pharmacologic (YC137) approaches in three breast cancer variants; MCF-7/LCC1 (ICI sensitive), MCF-7/LCC9 (ICI resistant), and LY2 (ICI resistant). YC137 inhibits BCL-W and BCL2 and restores ICI sensitivity in resistant cells. Co-inhibition of BCL-W and BCL2 is both necessary and sufficient to restore sensitivity to ICI, and explains mechanistically the action of YC137. These data implicate functional cooperation and/or redundancy in signaling between BCL-W and BCL2, and suggest that broad BCL2 family member inhibitors will have greater therapeutic value than targeting only individual proteins. Whereas ICI sensitive MCF-7/LCC1 cells undergo increased apoptosis in response to ICI following BCL-W±BCL2 co-inhibition, the consequent resensitization of resistant MCF-7/LCC9 and LY2 cells reflects increases in autophagy (LC3 cleavage; p62/SQSTM1 expression) and necrosis but not apoptosis or cell cycle arrest. Thus, *de novo* sensitive cells and resensitized resistant cells die through different mechanisms. Following BCL-W+BCL2 co-inhibition, suppression of functional autophagy by 3-methyladenine or BECN1 shRNA reduces ICI-induced necrosis but restores the ability of resistant cells to die through apoptosis. These data demonstrate the plasticity of cell fate mechanisms in breast cancer cells in the context of antiestrogen responsiveness. Restoration of ICI sensitivity in resistant cells appears to occur through an increase in autophagy-associated necrosis. BCL-W, BCL2, and BECN1 integrate important functions in determining antiestrogen responsiveness, and the presence of functional autophagy may influence the balance between apoptosis and necrosis.

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Introduction

Approximately 70% of all newly diagnosed breast cancers express estrogen receptor-alpha (ER) [1], many of which are sensitive to antiestrogens. The steroidal antiestrogen ICI 182,780 (ICI; Faslodex, Fulvestrant) is a selective ER downregulator (SERD) that acts as an ER antagonist and enhances ubiquitin-mediated ER degradation. ICI is an effective second-line treatment for TAM resistant, ER-positive (ER+) tumors, and is as effective as some aromatase inhibitors [2,3]. One limitation of antiestrogen therapy is the prevalence of *de novo* and acquired resistance in breast cancer. Acquired antiestrogen resistance occurs when a tumor has an initially beneficial response to antiestrogen treatment but the remaining tumor cells stop responding [4,5]. We report the roles of BCL2L2 (BCL-W), BCL2, and Beclin-1 (BECN1) in affecting responsiveness to ICI-resistance, and describe how anti-apoptotic BCL2 family members are involved in determining breast cancer cell fate.

BCL2 family proteins are essential regulators of apoptosis. BCL2 and BCL-W are both antiapoptotic members of this family. BCL-W maintains cell viability by preventing mitochondrial membrane depolarization and caspase activation [6]. BCL-W acts by binding to pro-apoptotic BCL2 family members and preventing mitochondria-mediated apoptosis [7]. Overexpression of BCL-W can prevent cell death [6] but its role(s) in affecting breast cancer cell fate decisions or antiestrogen responsiveness is unknown. BCL2 also blocks the induction of apoptosis by inhibiting the activation of pro-apoptotic family members such as BAX and preventing mitochondrial membrane depolarization [8,9]. Overexpression of BCL2 is a potential mediator of resistance to several chemotherapeutic drugs [10].

BCL2 family members also play essential roles in autophagy (macroautophagy), a process characterized by the presence of autophagosomes that engulf damaged organelles for subsequent lysosomal degradation. Several anti-apoptotic BCL2 family members inhibit the activity of BECN1 [11], a key regulator of autophagy [12] that binds to PIK3C3 to facilitate autophagosome production [13]. However, the precise relationships between apoptosis and autophagy are unclear. Apoptosis or autophagy can each lead to cell death, but in some cellular contexts autophagy is a pro-survival process, for example, in the face of nutrient deprivation [11]. While autophagy can contribute to TAM resistance in some

breast cancer cells [14–16], its role in response to other antiestrogens is unknown. In ER+ MCF-7 breast cancer cells treated with camptothecin, autophagy prolongs survival and delays apoptosis [17]. In marked contrast, autophagy promotes apoptosis in MCF-7 cells treated with the cytotoxic diterpenoid oridonin, where an inhibition of autophagy increases cell survival [18].

We determined whether BCL-W and BCL2 regulate ICI response in human breast cancer cells, and whether any effects involve changes in apoptosis and/or BECN1-associated autophagy. We used three estrogen-independent cell lines: MCF-7/LCC1 (ICI sensitive) [19], and LY2 and MCF-7/LCC9 cells that are crossresistant to TAM and ICI [20,21]. We show that co-inhibition of BCL-W and BCL2 restores sensitivity to the growth-inhibitory effects of ICI in both MCF-7/LCC9 and LY2 cells. In re-sensitized cells, ICI treatment increases the levels of autophagy and necrosis but has no effect on apoptosis. Inhibition of autophagy by 3-methyladenine (3MA) or BECN1 shRNA under these conditions reduces necrosis and increases apoptosis. Thus, restoration of ICI sensitivity with BCL-W+BCL2 inhibition appears to occur through increasing an autophagy-associated necrotic cell death. Finally, we show that co-inhibiting BCL-W and BCL2 improves ICI sensitivity in antiestrogen-sensitive cells by increasing apoptosis. Therefore, BCL-W, BCL2, and BECN1 integrate central functions in determining ICI responsiveness likely by regulating functional autophagy to dictate the balance between apoptotic and necrotic cell death.

Results

We measured endogenous BCL-W and BCL2 expression in control and ICI treated resistant and sensitive cells. BCL2 expression was significantly higher in ethanol control and ICI treated (resistant) MCF-7/LCC9 cells when compared to (sensitive) MCF-7/LCC1 cells (**Figure 1A**; ANOVA $p = 0.002$). BCL-W expression was lower in MCF-7/LCC1 cells after 24 hr of ICI treatment and increased in both sensitive and resistant cells after 72 hr of ICI treatment. However, the levels in resistant cells remained higher than in sensitive cells (**Figure 1B**; ANOVA $p = 0.004$;).

To determine if BCL2 transcription is regulated in antiestrogen-resistant cells, we measured basal BCL2 promoter activity using a BCL2-luciferase promoter-reporter assay. Basal BCL2 promoter activity was increased 14-fold in MCF-7/LCC9 cells (**Figure S1**;

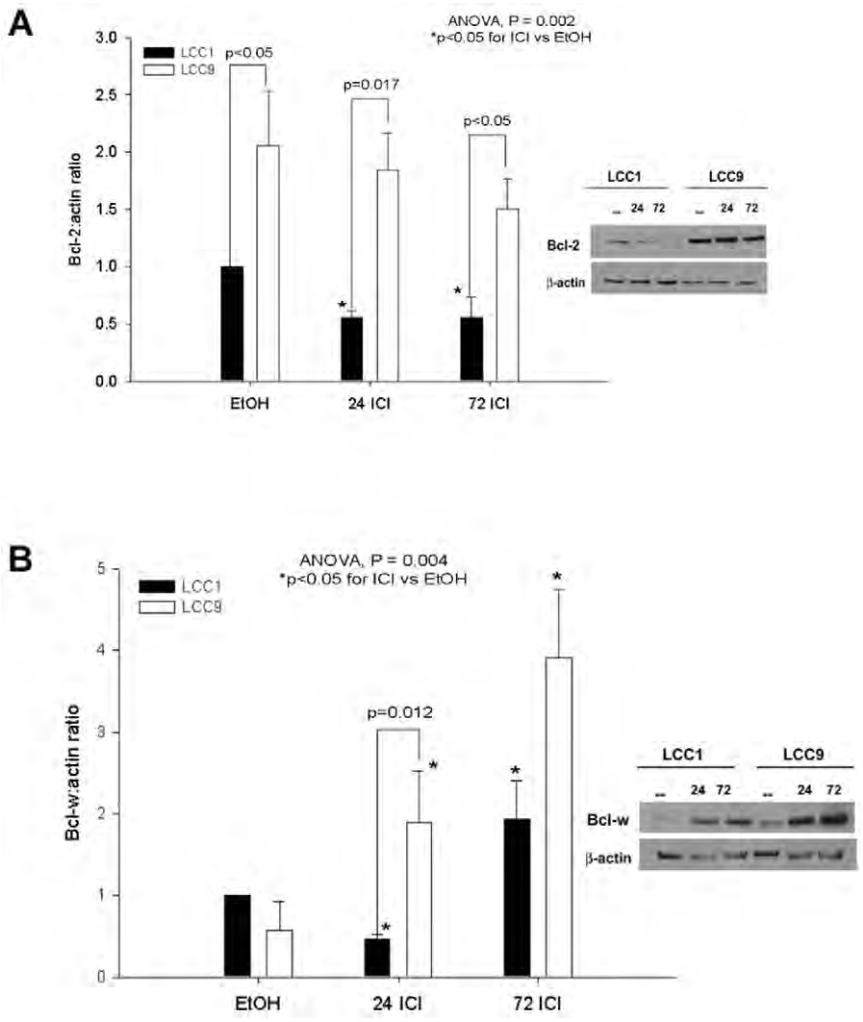

Figure 1. Increased expression of BCL-W and BCL2 in MCF-7/LCC9 cells. Whole cell lysates were subjected to Western blot analysis with a specific BCL2 or BCL-W antibody. (**A**) Bars represent the mean±SE of the relative BCL2:actin ratio (normalized to control cells) for three independent experiments. *Inset, a representative blot.* (**B**) Bars represent the mean±SE of the relative BCL-W:actin ratio (normalized to control cells) for three independent experiments. *Inset, a representative blot.*
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$p<0.003$) when compared to MCF-7/LCC1 cells, suggesting that the transcriptional regulation of basal BCL2 expression is altered in MCF-7/LCC9 cells.

YC137 Restores ICI 182,780 Sensitivity by Increasing Necrotic but Not Apoptotic Cell Death in Antiestrogen Resistant Cells

We hypothesized that if the expression of pro-survival BCL2 family members is responsible for the resistance phenotype its inhibition should restore antiestrogen sensitivity. We first tested this hypothesis using the small molecule BCL2 inhibitor YC137 [22]. MCF-7/LCC1 and MCF-7/LCC9 cells were treated with YC137 (400 nmol/L) and ICI (20 nmol/L and 500 nmol/L) for 7-days. Total cell number was significantly decreased after treatment with both concentrations of ICI and/or YC137 in MCF-7/LCC1 cells (ANOVA $p<0.001$; Fig. 2*A*). RI = 2.56 (20 nmol/L ICI) and RI = 1.23 (500 nmol/L ICI) suggest a strong synergistic interaction between 20 nmol/L ICI and YC137; the weaker interaction between 500 nmol/L ICI and YC137 reflects the high potency of 500 nmol/L ICI alone in sensitive cells. In resistant cells, neither ICI nor YC137 alone affected cell proliferation, whereas total cell number decreased after YC137+ICI treatment (**Figure 2A**; ANOVA $p = 0.001$) indicating a restoration of ICI sensitivity; RI = 1.56 for the YC137+ICI treatment implies a synergistic interaction.

To determine the effect of BCL-W and BCL2 inhibition in sensitive cells, MCF-7/LCC1 cells were treated with increasing

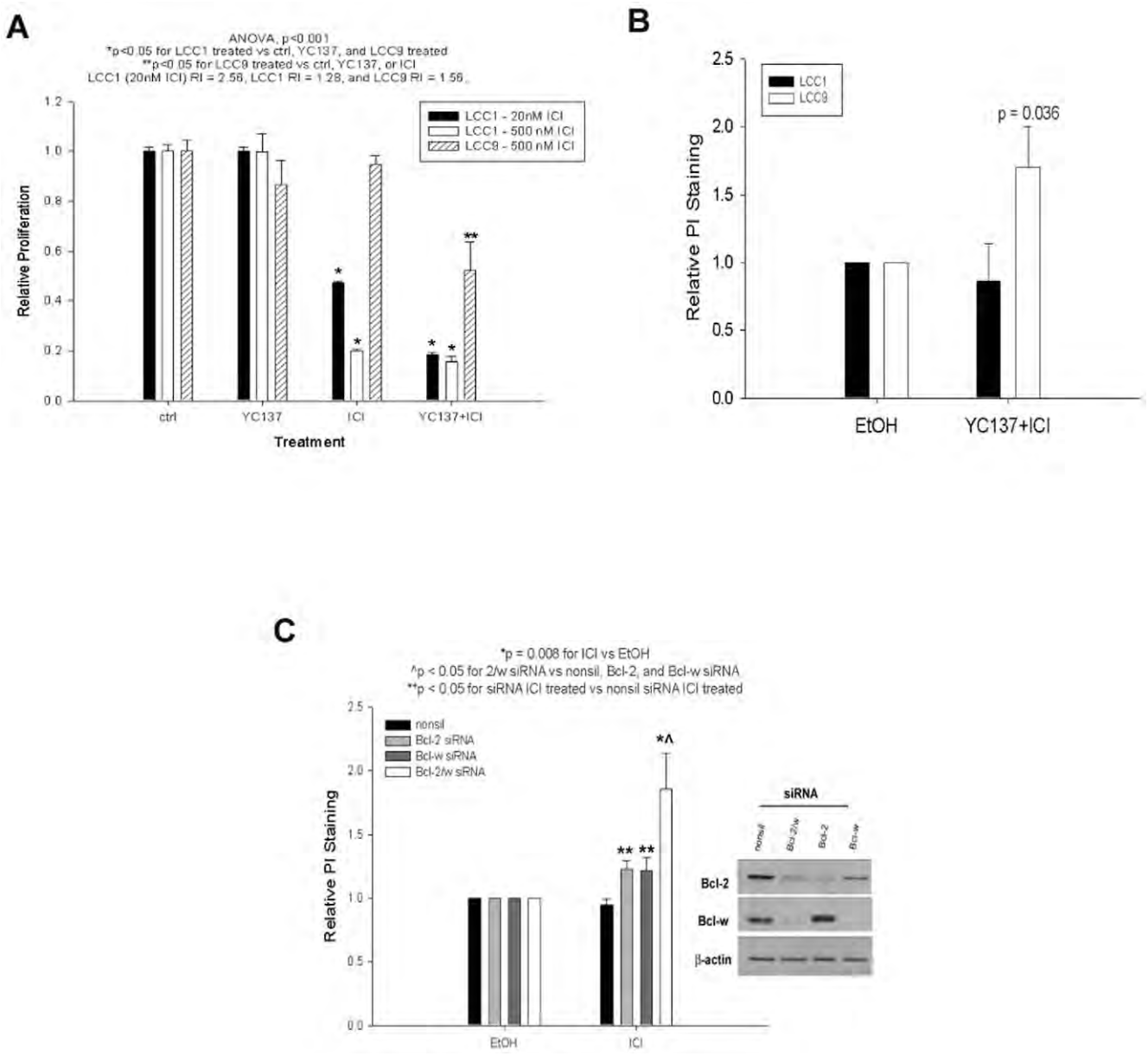

Figure 2. BCL-W and BCL2 inhibition increases sensitivity to ICI 182,780 and increases necrosis in MCF-7/LCC9 cells. (**A**) Cells were treated with YC137 and/or ICI for 7-days. Bars represent the mean±SE of relative cell proliferation (normalized to EtOH treated controls) for a single representative experiment performed in triplicate. (**B**) Cells were treated and stained with propidium iodide (PI). Bars represent the mean±SE of relative PI staining (normalized to control EtOH treated cells) for three independent experiments. (**C**) Cells were transfected with siRNA and stained with PI. *Inset, a representative blot showing BCL-W and BCL2 siRNA knockdown.*
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concentrations of YC137. Five days of YC137 treatment had no effect; however, cell proliferation decreased significantly after 7-days (**Figure S2A**; ANOVA $p<0.001$). After 5-days of YC137+ICI treatment, YC137 further decreased cell proliferation after treatment with ICI (**Figure S2B**; ANOVA $p<0.001$). Results with 20 nM ICI are included in Fig. 2*A* for comparison.

To determine if YC137 increases apoptosis, MCF-7/LCC1 and MCF-7/LCC9 cells were treated with YC137+ICI for 48 hr. Cell fate was evaluated by measuring FITC-Annexin V (apoptosis) and propidium iodide (PI) staining (to measure necrosis; not to detect the sub-G_1 peak) by FACS. In contrast to MCF-7/LCC1 cells, treatment of MCF-7/LCC9 cells with YC137 or ICI only, or YC137+ICI did not induce apoptosis (not shown). However, in MCF-7/LCC9 cells treated with YC137+ICI a significant increase in PI staining was observed (**Figure 2B**; $p = 0.036$).

Whether the effects of YC137 are driven by inhibition of BCL-W, BCL2, or inhibition of both proteins is required, is unknown. To determine the effects of specific BCL2 family members on the changes in cell death seen with YC137 treated cells, BCL-W and BCL2 siRNA were used individually or concurrently to inhibit their expression. Knockdown of either BCL-W or BCL2 individually or in combination in MCF-7/LCC9 cells does not result in increased apoptosis when combined with ICI treatment in resistant cells (not shown). However, we detected a significant increase in PI staining after BCL-W±BCL2 knockdown and ICI treatment (**Figure 2C**; $p<0.05$), the greatest effect was seen when both BCL-W and BCL2 are co-inhibited (**Figure 2C**; $p<0.05$). To confirm these observations morphologically, cells were treated for 48 hr prior to staining with an acridine orange/ethidium bromide solution and examined by fluorescence microscopy. Images of viable cells (large, green nuclei), apoptotic cells (condensed, green nuclei), late apoptotic cells (condensed red nuclei), and necrotic cells (large, red-orange nuclei) were captured. The greatest proportion of necrotic cells is seen with YC137+ICI treatment (data not shown). These data show that BCL-W+BCL2 co-inhibition in ICI treated antiestrogen resistant cells most strongly increases necrosis without significantly altering the rate of apoptosis, while inhibition of BCL2 or BCL-W alone is not sufficient.

ICI 182,780 Treatment Combined with BCL2 and BCL-W Inhibition Increases Autophagy in Resistant Cells

During autophagy LC3 is cleaved to form LC3I and LC3II, whereas p62/SQSTM1 binds to LC3 and is degraded [23]. To determine if YC137 treatment acts by increasing autophagy, as might be expected from its inhibition of BCL2 [24], cells were treated with YC137 and/or ICI and examined for LC3 cleavage and p62/SQSTM1 expression by Western blotting. In MCF-7/LCC9 cells, there was a significant increase in LC3II expression after YC137+ICI treatment when compared to ethanol treated controls and MCF-7/LCC1 cells (**Figure 3A**; ANOVA $p<0.001$). LC3II expression in the combination-treated cells was also significantly higher than in cells treated with either YC137 or ICI alone (**Figure 3A**; ANOVA $p<0.001$). Consistent with the predicted increase in autophagy, p62/SQSTM1 expression was downregulated in MCF-7/LCC9 cells treated with YC137 or YC137+ICI; expression in the combination treated cells was significantly lower than in cells treated with YC137 or ICI alone (**Figure 3B**; ANOVA $p<0.024$). This decrease in p62/SQSTM1 expression was also observed in YC137+ICI treated MCF-7/LCC1 cells (**Figure 3B**; ANOVA $p<0.024$). To determine if BCL2+BCL-W knockdown produces the same effect as YC137, MCF-7/LCC9 cells were transfected with both BCL2 and BCL-W siRNAs and treated with ICI. Consistent with the effects of YC137, BCL-W and BCL2 co-inhibition significantly increased LC3II expression after ICI treatment (**Figure 3C**; $p<0.05$).

Combined Inhibition of BCL2, BCL-W, and Autophagy (by 3MA) Increases Apoptosis and Decreases Necrosis

To investigate the functional role of autophagy after BCL-W and BCL2 co-inhibition, MCF-7/LCC9 cells were treated with the autophagy inhibitor 3MA (350 μmol/L) in combination with ICI and YC137. Cell number was significantly decreased in 3MA+ICI+YC137 co-treated cells (**Figure 4A**; ANOVA $p<0.001$). However, treatment with 3MA+ICI+YC137 did not decrease further MCF-7/LCC9 cell proliferation when compared to ICI+YC137 (**Figure 4A**).

Autophagy can be pro-death [15,18] or pro-survival [17,25]. To determine the effect of autophagy inhibition on cell death, we measured mitochondrial membrane permeability (MMP), apoptosis, and necrosis after treatment with 3MA. The ICI-resistant LY2 cells were also examined to compare their response to BCL2+BCL-W co-inhibition and autophagy inhibition with ICI-resistant MCF-7/LCC9 cells. While LY2 cells express low basal levels of BCL-W, BCL2, and LC3II (not shown), cell proliferation was significantly down-regulated following YC137, ICI, and YC137+ICI treatment; proliferation is lowest in combination treated cells when compared to the individual treatments (**Figure S3**; ANOVA, $p<0.001$). Following treatment with 3MA+YC137+ICI, Annexin V staining increased significantly in resistant cells (MCF-7/LCC9; LY2) when compared to controls, cells treated with each of 3MA, YC137, or ICI alone, or YC137+ICI (**Figure 4B**; ANOVA $p<0.001$). MCF-7/LCC1 cells increased relative Annexin V staining after all treatments except when treated with 3MA alone (**Figure 4B**; $p<0.001$).

We then determined if these effects were associated with changes in the mitochondria. Consistent with the Annexin V staining, MMP increased significantly in MCF-7/LCC1 cells following treatment with ICI and YC137 alone, and after treatment with ICI combined with YC137 and/or 3MA. In contrast, MCF-7/LCC9 cells exhibit increased MMP only after treatment with 3MA+YC137+ICI (**Figure 4C**; $p<0.001$). A decrease in PI staining, indicating a decrease in necrosis, occurred in resistant cells only after the addition of 3MA to YC137+ICI (**Figure 4D**; ANOVA $p<0.001$). There was no change in the number of cells in S-phase after treatment of MCF-7/LCC9 cells with YC137, 3MA, YC137+ICI, or 3MA+YC137+ICI (**Figure S4**). Thus, the reversal of resistance can occur without the cell cycle arrest seen in *de novo* sensitive cells. As expected, the number of MCF-7/LCC1 cells undergoing S-phase decreased after YC137+ICI and 3MA+YC137+ICI treatment (ANOVA, $p = 0.016$; **Figure S4**).

To establish further the roles of BCL-W and BCL2, we performed similar studies in BCL-W+BCL2 siRNA co-transfected resistant cells (MCF7/LCC9; LY2) treated with ICI and/or 3MA. After treating the siRNA transfected MCF-7/LCC9 cells with a combination of ICI and 3MA, we found a significant increase in Annexin V staining in BCL-W and BCL2 siRNA co-transfected cells (**Figure 5A**; ANOVA $p<0.001$). A significant decrease in PI staining was also observed after BCL-W/BCL2 knockdown in combination with ICI+3MA treatment (**Figure 5B**; ANOVA $p = 0.05$). These data imply that functional autophagy plays a major role in influencing the decision to undergo apoptosis and/or necrosis in antiestrogen-resistant cells.

BECN1 Mediates Key Effects of YC137, BCL-W, and BCL2

While basal BECN1 expression levels are comparable in sensitive and resistant cells (not shown), we could not exclude

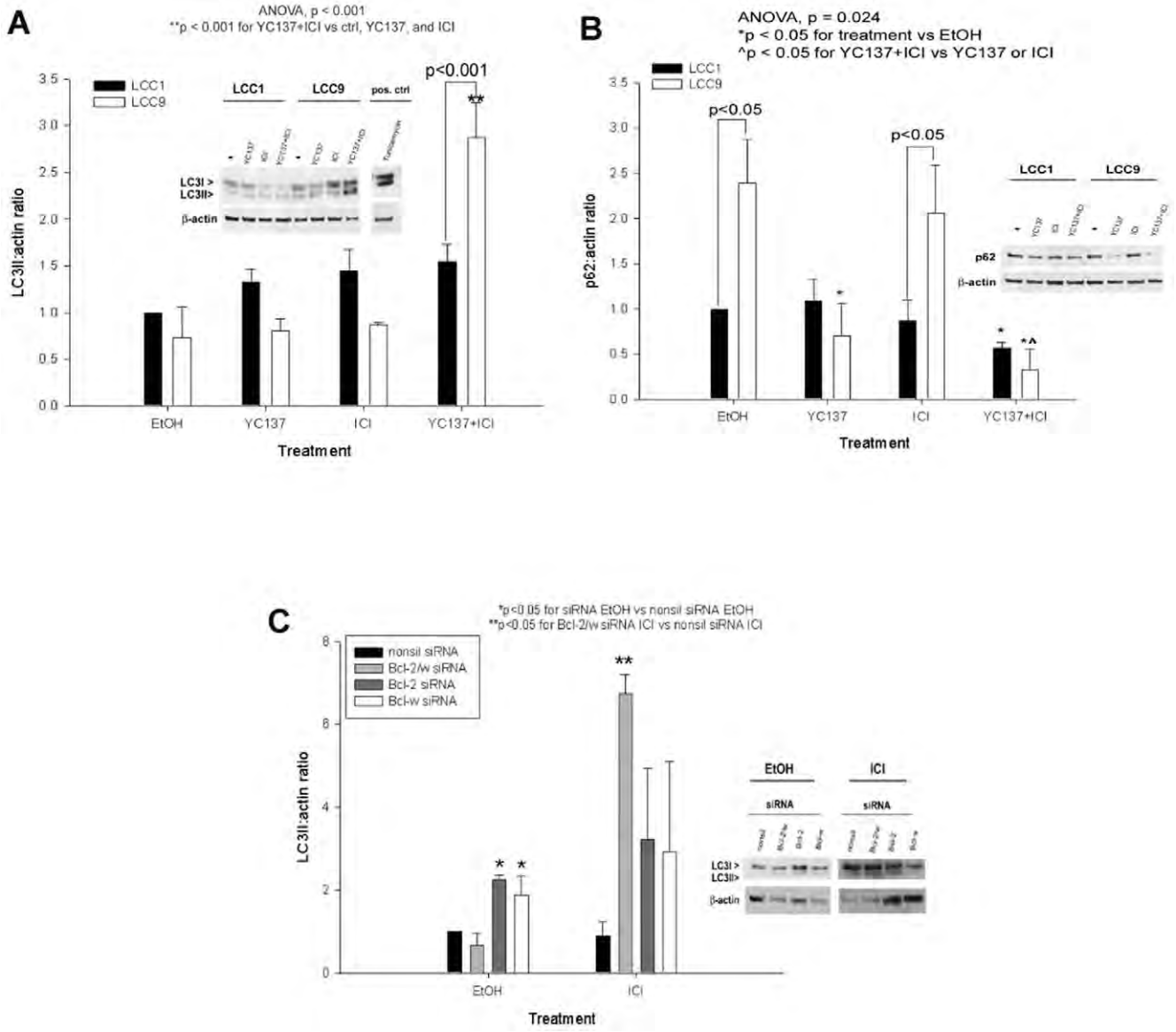

Figure 3. LC3II and p62/SQSTM1 expression after BCL-W and BCL2 inhibition. Whole cell lysates were subjected to Western blot analysis with a specific LC3 or p62/SQSTM1 antibody. (**A**) Bars represent the mean±SE of the relative LC3II:actin ratio (normalized to empty vector controls) for three independent experiments. *Inset, a representative blot*. (**B**) Bars represent the mean±SE of the relative p62/SQSTM1:actin ratio (normalized to empty vector controls) for three independent experiments. *Inset, a representative blot*. (**C**) Cells were transfected with siRNA and LC3II measured by Western blot analysis. Bars represent the mean±SE of the relative LC3II:actin ratio (normalized to empty vector controls) for three independent experiments.

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the possibility that its role is functionally different in these cellular contexts. Thus, we explored the mechanistic relationship between BCL-W, BCL2, and BECN1 using shRNA-mediated knockdown of BECN1 in resistant cells. BECN1 shRNA effectively decreased BECN1 protein expression (approximate 5-fold) in MCF-7/LCC9 cells (**Figure 6A**; $p = 0.029$). Also, BECN1 shRNA infected MCF-7/LCC9 cells were more sensitive to YC137 (50 nmol/L) and ICI (500 nmol/L) than control infected cells, and cell proliferation was down-regulated after treatment with YC137 or ICI (**Figure 6B**; ANOVA, $p<0.001$). However, cell proliferation following YC137+ICI treatment was significantly lower than either treatment alone. For BECN1 knockdown combined with ICI treatment, $RI = 1.23$ suggests at least an additive interaction. Unlike control infected cells, proliferation was downregulated in BECN1 shRNA infected cells treated with YC137 or YC137+ICI (**Figure 6B**; ANOVA $p<0.001$). Furthermore, apoptosis was significantly increased in cells treated with YC137+ICI (**Figure 6C**; ANOVA $p<0.001$). The level of necrosis increased in control infected cells, and decreased in BECN1 shRNA infected cells, when treated with YC137+ICI (**Figure 6D**; ANOVA $p<0.05$). BECN1 knockdown, in combination with BCL2+BCL-W co-inhibition, inhibited autophagy, restored ICI sensitivity, and increased apoptosis (but not necrosis) in ICI treated antiestrogen-resistant breast cancer cells. These different cell death outcomes in sensitive and resistant cells indicate considerable plasticity in breast cancer cell fate mechanisms in response to antiestrogens.

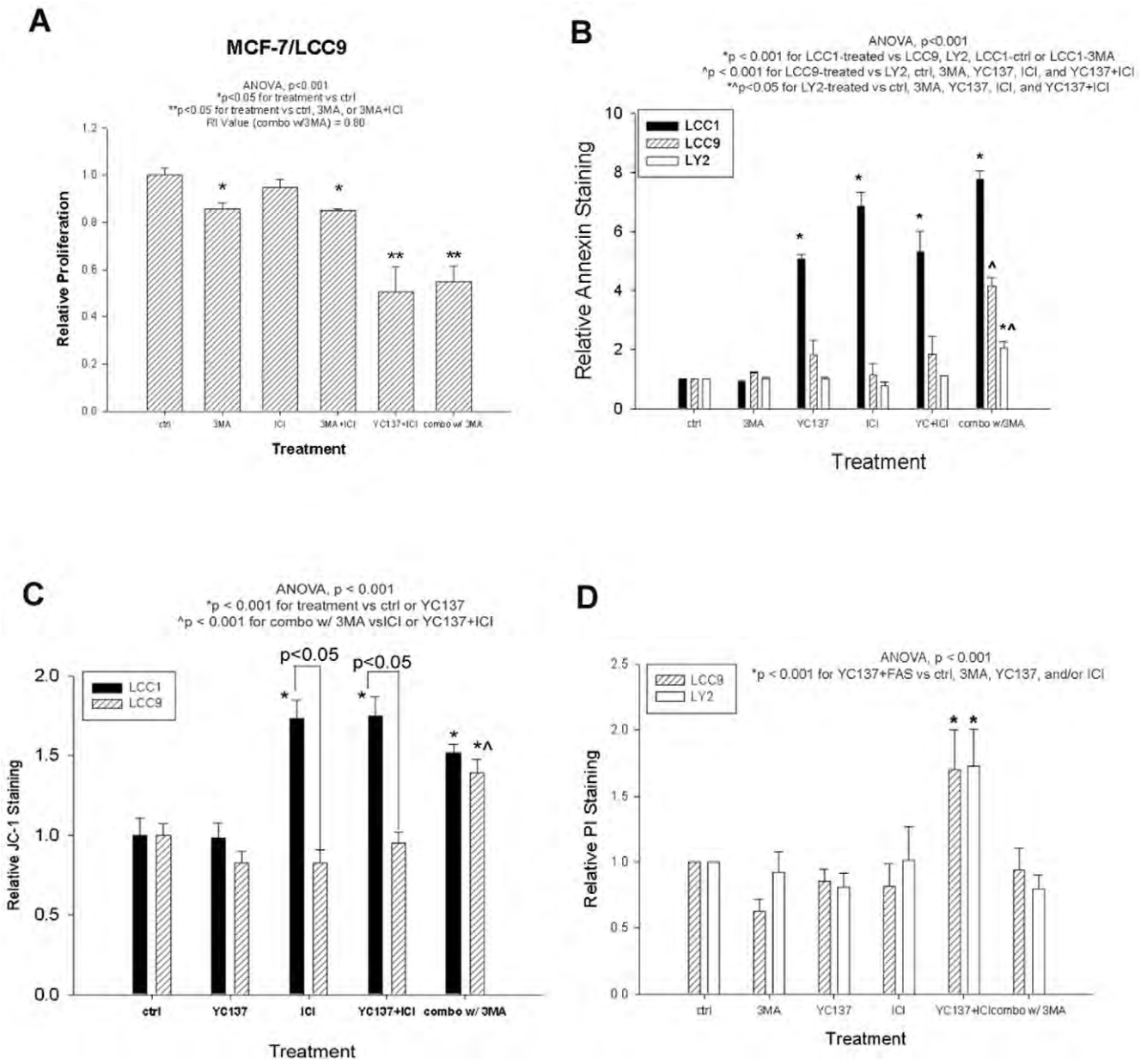

Figure 4. Increased apoptosis and decreased necrosis after BCL-W and BCL2 and autophagy inhibition. (**A**) Cells were treated with ICI, 3MA, YC137, or a combination of the three for 7-days. Bars represent the mean±SE of relative proliferation (normalized to empty vector control). (**B**) Cells were treated with YC137, ICI, 3MA, YC137+ICI, or a combination of YC137, ICI, and 3MA for prior to Annexin V staining. Bars represent the mean±SE of the relative Annexin V staining (normalized to empty vector controls) for three independent experiments. (**C**) Cells were treated with ICI, 3MA, YC137, or a combination prior to JC-1 staining. (**D**) Cells were treated with YC137, ICI, 3MA, YC137+ICI, or a combination of YC137, ICI, and 3MA prior to PI staining. Bars represent the mean±SE of the relative PI staining (normalized to empty vector controls) for three independent experiments.
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Discussion

Antiestrogen resistance is a major limitation to improving breast cancer survival rates and elucidating its mechanisms remains an important challenge [26,27]. In breast tumors, BCL2 expression measured prior to therapy correlates with ER expression and an improved response to antiestrogens [28]. However, BCL2 levels decrease after TAM therapy, but only in those women who obtain clinical benefit [29]. In breast tumors, apoptosis increases after the first 24 hr of TAM treatment but markedly decreases 3-months later. Moreover, BCL2 expression is elevated in residual (resistant) tumors [30]. We hypothesized that increased expression of BCL2 and/or BCL-W may play a role in antiestrogen resistance by allowing resistant cells to evade apoptosis. We show that in the absence of estrogen there is an increase in basal and ICI-regulated BCL2 mRNA, protein, and promoter activity in resistant cells, observations consistent with data showing elevated activity of two upstream regulators of BCL2: NFκB and XBP1 [31–33]. However, co-inhibition of BCL-W and BCL2 is required to restore ICI sensitivity, a process that is driven by increased autophagy and necrosis, but not apoptosis. We also show that increased autophagy may activate necrotic cell death in resistant cells.

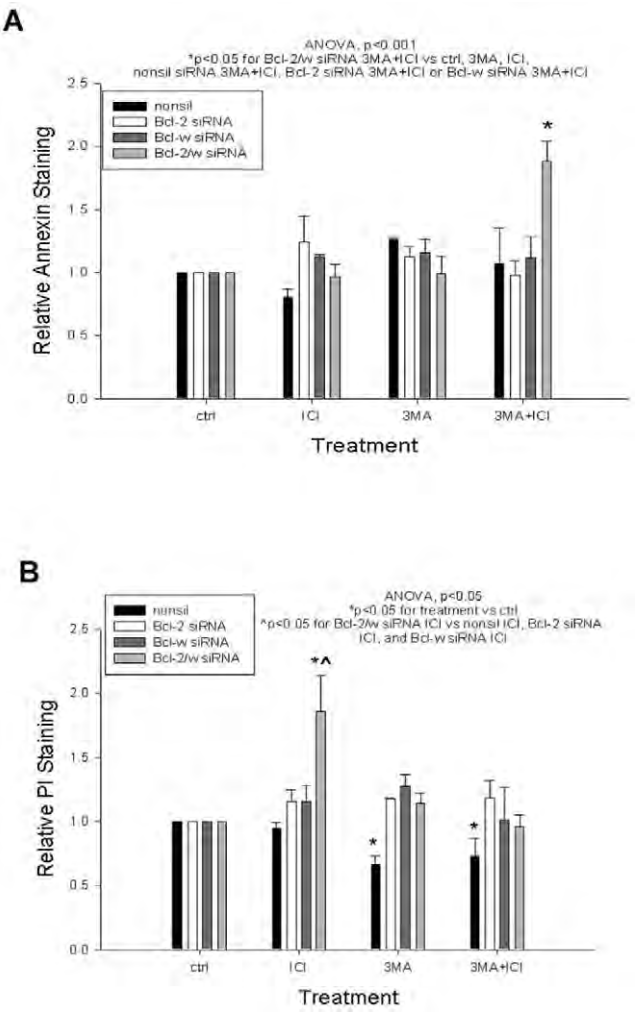

Figure 5. BCL-W/BCL2 knockdown and autophagy inhibition increases apoptosis and decreases necrosis. (**A**) MCF-7/LCC9 cells were transfected with a combination of BCL-W and BCL2 siRNA and treated with ICI and 3MA. Bars represent the mean±SE of the relative Annexin V staining (normalized to empty vector controls) for three independent experiments. (**B**) Bars represent the mean±SE of the relative PI staining (normalized to empty vector controls) for three independent experiments.
doi:10.1371/journal.pone.0008604.g005

Little is known about BCL-W expression and function in breast cancer. Since BCL-W is overexpressed in some human colon cancer cells [6,34] and its expression is regulated by estrogen in cerebrocortical neuron cultures [35], we hypothesized that BCL-W could play a role in antiestrogen resistance. BCL-W expression is increased by ICI in both sensitive and resistant cells, suggesting that an increased co-expression of both BCL2 and BCL-W is required for antiestrogen resistance. Hence, the ICI-induced increase in the expression of BCL-W alone in antiestrogen-sensitive cells has little effect on responsiveness unless accompanied by a concurrent increase in BCL2, as is seen in resistant cells.

Small-molecule inhibitors of proapoptotic BCL2 family members can restore sensitivity to some therapeutic agents that induce apoptosis [36]. Some of these compounds inhibit the proliferation of cells that express high levels of BCL2 [37]. However, several antiapoptotic BCL2 family members also regulate autophagy through their interactions with BECN1 [12,38,39]. In resistant MCF-7/LCC9 and LY2 cells, only the levels of autophagy and necrosis increase after YC137+ICI treatment; there is no increase in either MMP or apoptosis.

We used BCL-W and/or BCL2 siRNA to confirm the results with YC137. As expected, BCL-W+BCL2 co-inhibition has no effect on apoptosis in ICI treated MCF-7/LCC9 cells, whereas both autophagy and necrosis increase. Inhibition of BCL2 and BCL-xL decreases cellular ATP and increases necrosis (but not apoptosis) in acinar cells hyperstimulated with CCK-8 [40]. Autophagy can also activate necrosis in apoptosis-deficient mouse embryonic fibroblasts [41]. Increased autophagosome formation is induced early during necrotic cell death and contributes to the cellular destruction that occurs during necrosis in *Caenorhabditis elegans* [42]. These results suggest that BCL-W+BCL2 coinhibition can increase antiestrogen sensitivity in resistant breast cancer cells by preferentially activating necrosis, apparently in association with the induction of autophagy. In contrast, inhibiting autophagy in some TAM-resistant breast cancer cells can increase apoptosis [16].

No change occurs in the proportion of cells undergoing S-phase after 3MA+YC137+ICI treatment. Thus, it is unlikely that autophagy plays a major role in the cell cycle arrest effects of antiestrogens. We also show that the inhibition of autophagy, in combination with BCL-W+BCL2 co-inhibition in ICI treated resistant cells, does not further reduce total cell number but shifts programmed cell death such that apoptosis increases and necrosis decreases. Our results strongly suggest that functional autophagy is a central component of the cell fate decision machinery in ICI-resistant breast cancer cells, although we cannot exclude the possibility that autophagy also alters the kinetics of cell death. Nonetheless, in addition to being a cell death effector mechanism, autophagy appears to be a central component in influencing how breast cancer cells die in response to antiestrogens.

In summary, our results show that BCL-W+BCL2 co-inhibition restores ICI sensitivity in antiestrogen-resistant cells and increases ICI sensitivity in antiestrogen-sensitive cells. We show that the overexpression of BCL-W and BCL2 is linked to determining cell fate through autophagy in ICI resistant breast cancer models (**Figure S5A**). We have shown that BCL-W+BCL2 coinhibition increases autophagy and necrosis with no effect on the extent of apoptotic cell death (**Figure S5B**). These data suggest that BCL-W and BCL2 activate apoptosis and necrosis by initially regulating autophagy (**Figure S5C**). We conclude that the co-inhibition of BCL-W and BCL2 restores sensitivity in antiestrogen-resistant breast cancer cells by promoting an autophagy-associated increase in necrosis. Antiestrogen sensitive cells undergo autophagy and/or apoptosis, whereas resistant cells undergo autophagy and necrosis when resensitized. These different cell death outcomes in sensitive and resistant cells show the notable plasticity of cell fate mechanisms in breast cancer. In resistant cells, resensitization to antiestrogens can also occur without the cell cycle arrest that accompanies cell death in *de novo* sensitive cells. Thus, antiestrogen-regulated signaling that modifies cell cycling occurs through mechanisms independent of mitochondrial function and cell death.

From a therapeutic perspective, these data also suggest that broad rather than specific BCL2 family member inhibitors will have greater clinical value and may explain the apparent lack of activity of targeted BCL2 antisense monotherapy in clinical trials [43]. Combination therapy with endocrine agents and broadly active small molecule inhibitors of BCL2 family members may delay, prevent, or reverse the acquisition of antiestrogen resistance in breast cancer patients and lead to significant improvements in survival.

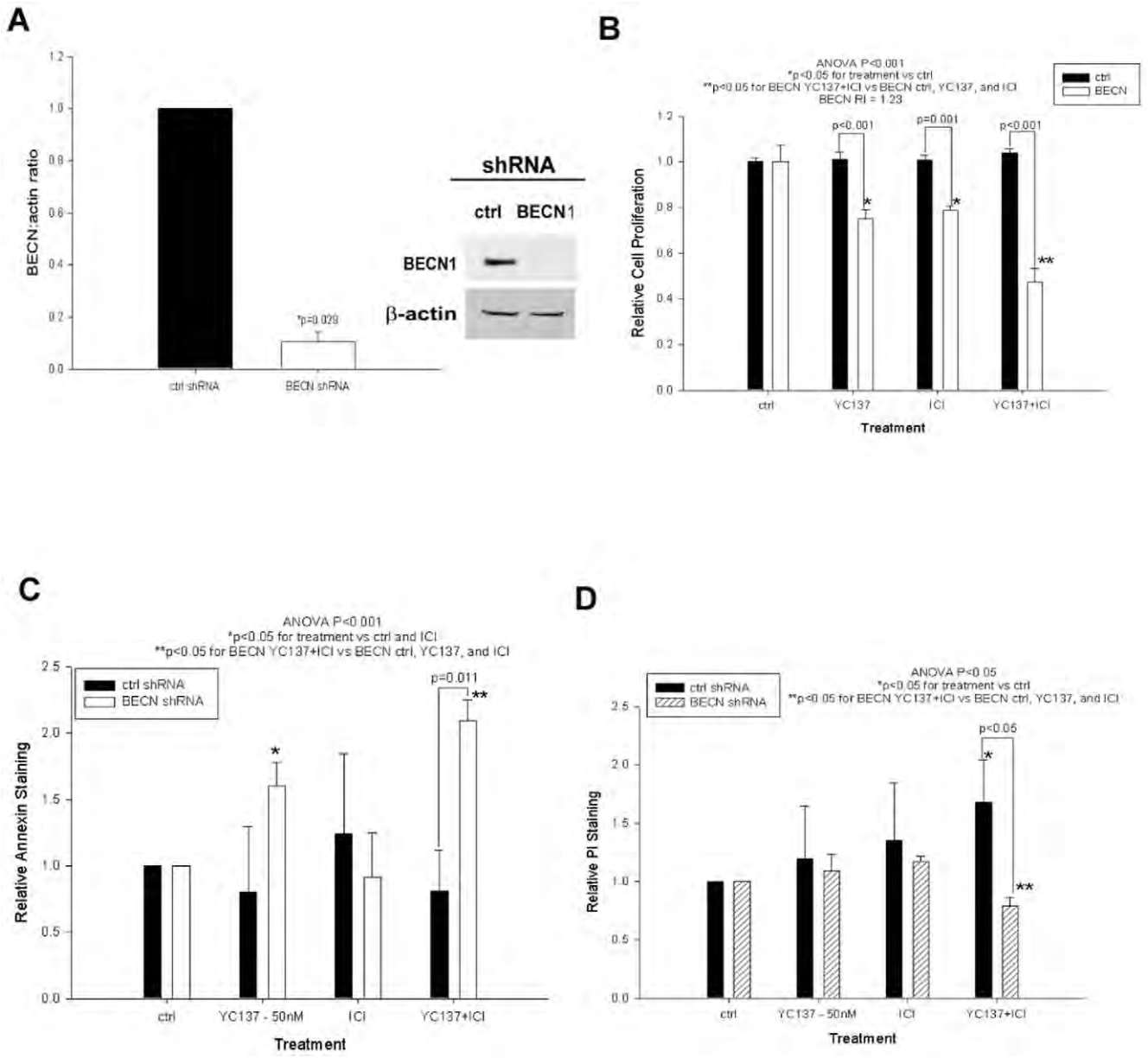

Figure 6. BECN1 knockdown and BCL-W/BCL2 co-inhibition decreases cell proliferation through increased apoptosis in resistant cells. (**A**) Whole cell lysates were subjected to Western blot analysis with a specific BECN1 monoclonal antibody. Bars represent the mean±SE of the relative BECN1:actin ratio (normalized to control cells) for three independent experiments. (**B**) shRNA infected MCF-7/LCC9 cells were treated with YC137 and/or ICI for 7-days. Bars represent the mean±SE of relative cell proliferation (normalized to EtOH treated controls) for a single representative experiment performed in triplicate. (**C**) shRNA infected MCF-7/LCC9 cells were treated with YC137, ICI, or a combination of YC137 and ICI. Bars represent the mean±SE of the relative Annexin V staining (normalized to empty vector controls) for three independent experiments. (**D**) Bars represent the mean±SE of the relative PI staining (normalized to empty vector controls) for three independent experiments.
doi:10.1371/journal.pone.0008604.g006

Materials and Methods

Cell Culture

All cells were shown to be free of *Mycoplasma* spp. contamination. MCF-7/LCC1 (ER+, estrogen independent, antiestrogen-sensitive) [19]; MCF-7/LCC9 (ER+, estrogen independent, TAM and ICI cross-resistant variant derived from MCF-7/LCC1 cells by selection against ICI) [21], and LY2 cells (ER+, estrogen independent, LY 117018, TAM, and ICI cross-resistant, MCF-7 variant derived by selection against the Raloxifene analog LY 117018) [20] were routinely grown in improved minimal essential medium without phenol red and supplemented with 5% charcoal stripped calf serum (CCS-IMEM; Biofluids). We confirmed the genetic lineage of the three variant cell lines as being derived from the original MCF-7 cell line by DNA fingerprinting using genetic markers at nine different loci. All cells were maintained at 37°C in a humidified incubator with 95% air:5% CO_2 atmosphere. ICI was obtained from Tocris Bioscience (Ellisville, MO) and 3-methyladenine (3MA) from Sigma Aldrich (St. Louis, MO). Acridine orange was obtained from EMD Biosciences (San Diego, CA) and ethidium bromide from Invitrogen (Carlsbad, CA). YC137 was kindly provided by Dr. York Tomita (Georgetown University) [44].

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using the Trizol method. For each cDNA sample a qPCR reaction and a standard curve were established using TaqMan Universal PCR Master Mix and the following TaqMan primers (Applied Biosystems): BCL2 = Hs00608023_m1; BCL-W (BCL2L2) = Hs00187848_m1; RPLP0 (housekeeping gene) = Hs99999902_m1. Each reaction (10 µl) was run in triplicate on an ABI Prism 7900HT Sequence Detection System using the manufacturer's absolute quantification protocol. Expression data for each reaction was estimated relative to expression of RPLP0.

Transient Transfection and Promoter-Reporter Assays

Cells were plated at 60,000 cells/well and maintained for 24 hr prior to co-transfection with 0.4 µg of full length BCL2 promoter-luciferase reporter plasmid [45] (a generous gift from Dr. Linda Boxer, Stanford University Medical Center) and 0.004 µg of the phRL-SV40-Renilla control plasmid containing the *Renilla* luciferase gene (Promega, Madison, WI). Activation of the BCL2 promoter was measured using the Dual Luciferase Assay Kit (Promega) and luminescence measured using a Lumat LB 9501 luminator (EG&G Berthold, Bundoora, Australia).

siRNA Transfection and Lentiviral shRNA Infection

Cells were plated at 100,000 cells/well and BCL2, BCL-W (Dharmacon, Lafayette, CO), and control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were each diluted to 100 nM. Transfection was performed according to Dharmacon's protocol using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with ICI, 3MA, a combination of the two, or ethanol vehicle for 48 hr. For the lentiviral infection, cells were plated at 10,000 cells/well and allowed to incubate for 24 hr prior to shRNA infection. BECN1 lentiviral particles and control lentiviral particles were purchased from Dharmacon. The infection was carried out according to the Dharmacon SMARTvector shRNA lentiviral protocol using Polybrene (Millipore).

Western Blotting

Cells were treated as appropriate and lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche) and 1 mmol/L sodium orthovanadate phosphatase inhibitor (Sigma). The primary antibodies used were: mouse monoclonal BCL2 primary antibody (1:1000; Assay Designs, Ann Arbor, MI), rabbit monoclonal BCL-W primary antibody (1:500; Cell Signaling, Danvers, MA), rabbit polyclonal LC3B primary antibody (1:500; Cell Signaling), mouse monoclonal p62/SQSTM1 primary antibody (1:500; Abcam, Cambridge, MA) overnight. Antigen-antibody complexes were visualized using the ECL detection system (Amersham Biosciences) and SuperSignal Chemiluminescent Substrate (Thermoscientific). Protein expression was quantified using densitometric analysis; data (mean±SE) are presented as the ratio of target protein: βactin signals.

Cell Proliferation

5,000 cells/well were treated as appropriate for 7-days. Following treatment, cells were stained with a crystal violet staining solution [46]. Sodium citrate buffer was added to each well and absorbance measured at 550 nM using a microplate reader (Biorad, Hercules, CA).

Cell Cycle, Apoptosis, Necrosis, and Autophagy

Fluorescence activated cell sorting (FACS) was performed by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource. For cell cycle analysis, cells were plated at 80,000–100,000 cells/well, treated as appropriate for 48 hr, fixed, and analyzed by FACS. To measure apoptosis, cells were treated for 48 hr and stained as described in the TACS Annexin V Kit (Trevigen, Gaithersburg, MD). Necrosis was measured by counting cells stained red by propidium iodide (PI). For morphologic analysis of necrosis, cells were plated, treated 24 hr later, and after a further 48 hr stained with acridine orange/ethidium bromide solution (100 µg/ml acridine orange in PBS:100 µg/ml ethidium bromide in PBS) and examined using an Olympus IX-70 confocal microscope with 488 nm and 633 nm excitation lasers.

To measure autophagy, we performed Western blot analysis to measure LC3 cleavage and p62/SQSTM1 expression [23]. Cells treated with 2 µg/ml tunicamycin (EMD Biosciences) for 48 hours were the positive control for LC3 cleavage. To block functional autophagy, we treated cells with the autophagy inhibitor 3MA, or infected cells with lentiviral BECN1 shRNA.

Mitochondrial Membrane Permeability

Cells were treated as appropriate and stained with 100 µl of JC-1 dye solution (Invitrogen) for 25 min at 37°C. Green fluorescence (485 nm excitation/535 nm emission) was measured on a Wallac Viktor2 1420 Multilabel Counter (Perkin-Elmer, Boston, MA).

Statistical Analyses

One-way ANOVA was used to determine overall significant differences following treatment in the cell proliferation, cell cycle, apoptosis, and MMP assays. Student's t-test was used to determine differences in BCL2, BCL-W, LC3, p62/SQSTM1 expression and luciferase promoter-reporter activity. All statistical analyses were performed using SigmaStat version 3.0. The nature of drug interactions (synergy, antagonism, additivity) was assessed using the Relative Index (RI) [47]. RI values were obtained by calculating the expected cell survival (S_{exp}; the product of survival obtained with drug A alone and the survival obtained with drug B alone) and dividing this S_{exp} by the observed cell survival in the presence of both drugs (S_{obs}). $S_{exp}/S_{obs}>1.0$ indicates a synergistic interaction, <1.0 indicates an antagonistic interaction, and $=1$ is indicative of an additive interaction between the two drugs used.

Supporting Information

Figure S1 Increased basal BCL2 promoter activity in ICI/TAM-cross-resistant MCF-7/LCC9 cells. Cells were seeded in 12-well plates and co-transfected with BCL2 promoter-luciferase and pCMV-Renilla constructs for 24 h prior to lysis and luminescent detection (to examine basal promoter activity). Bars represent the mean±SE of the relative BCL2-luciferase: Renilla luciferase activity for a single representative experiment performed in triplicate. $p<0.003$ for MCF-7/LCC9 vs. MCF-7/LCC1.
Found at: doi:10.1371/journal.pone.0008604.s001 (1.44 MB TIF)

Figure S2 Increased sensitivity to ICI 182,780 in antiestrogen-sensitive cells. A, MCF-7/LCC1 cells were treated with the indicated concentrations of YC137 for 5 and 7 days, at which time cell number was determined. Points represent the mean±SE of relative proliferation (normalized to empty vector control). ANOVA $p<0.001$; $p<0.05$ for YC137 vs. control. B, MCF-7/LCC1 cells were treated with ICI or a combination of YC137+ICI for 5 days, at which time cell number was determined. Points

represent the mean±SE of relative proliferation (normalized to empty vector control). ANOVA $p<0.001$; $p<0.001$ for YC137+ICI treated cells vs. ICI treated cells.
Found at: doi:10.1371/journal.pone.0008604.s002 (1.01 MB TIF)

Figure S3 BCL-W/BCL2 inhibition decreases cell proliferation in ICI 182,780 treated resistant LY2 cells. Cells were treated with ICI, YC137, or a combination of the two for 7 days to examine cell proliferation. Bars represent the mean±SE of relative proliferation (normalized to empty vector control). ANOVA $p<0.001$; $p<0.05$ for treatment vs. control treated cells and for YC137+ICI treated cells vs. YC137 and ICI.
Found at: doi:10.1371/journal.pone.0008604.s003 (0.66 MB TIF)

Figure S4 Combined autophagy inhibition and BCL-W/BCL-2 inhibition does not alter cell cycle distribution in the resistant cell line. Cells were treated with ICI, 3MA, YC137, or a combination for 48 h prior to ethanol fixation and FACS analysis. ANOVA $p = 0.016$; $p<0.05$ for treatment vs. control, 3MA, or YC137.
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Figure S5 BCL-W and BCL2 indirectly regulate necrosis through the direct regulation of autophagy and apoptosis. A, Representation of the relationship between BCL-W/BCL2 overexpression, autophagy, necrosis, and apoptosis in ICI-resistant cells treated with ICI 182,780. B, Representation of the effect of BCL-W/BCL2 inhibition on autophagy, necrosis, and apoptosis. C, Representation of the effect of BCL-W/BCL2 inhibition in combination with autophagy inhibition on autophagy, necrosis, and apoptosis.
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Author Contributions

Conceived and designed the experiments: ACC RBR ANS RC. Performed the experiments: ACC RBR ANS AZ. Analyzed the data: ACC RBR ANS AZ RC. Wrote the paper: ACC RBR ANS RC.

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Gene Network Signaling in Hormone Responsiveness Modifies Apoptosis and Autophagy in Breast Cancer Cells

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Abstract

Resistance to endocrine therapies, whether *de novo* or acquired, remains a major limitation in the ability to cure many tumors that express detectable levels of the estrogen receptor alpha protein (ER). While several resistance phenotypes have been described, endocrine unresponsiveness in the context of therapy-induced tumor growth appears to be the most prevalent. The signaling that regulates endocrine resistant phenotypes is poorly understood but it involves a complex signaling network with a topology that includes redundant and degenerative features. To be relevant to clinical outcomes, the most pertinent features of this network are those that ultimately affect the endocrine-regulated components of the cell fate and cell proliferation machineries. We show that autophagy, as supported by the endocrine regulation of monodansylcadaverine staining, increased LC3 cleavage, and reduced expression of p62/SQSTM1, plays an important role in breast cancer cells responding to endocrine therapy. We further show that the cell fate machinery includes both apoptotic and autophagic functions that are potentially regulated through integrated signaling that flows through key members of the BCL2 gene family and beclin-1 (BECN1). This signaling links cellular functions in mitochondria and endoplasmic reticulum, the latter as a consequence of induction of the unfolded protein response. We have taken a seed-gene approach to begin extracting critical nodes and edges that represent central signaling events in the endocrine regulation of apoptosis and autophagy. Three seed nodes were identified from global gene or protein expression analyses and supported by subsequent functional studies that established their abilities to affect cell fate. The seed nodes of nuclear factor kappa B (NFκB), interferon regulatory factor-1 (IRF1), and X-box binding protein-1 (XBP1) are linked by directional edges that support signal flow through a preliminary network that is grown to include key regulators of their individual function: NEMO/IKKγ, nucleophosmin and ER respectively. Signaling proceeds through BCL2 gene family members and BECN1 ultimately to regulate cell fate.

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Keywords

Antiestrogen; autophagy; apoptosis; breast cancer; cell signaling; endoplasmic reticulum; estrogens; gene networks; unfolded protein response

1. Introduction

Over 40,000 American women die of breast cancer each year [1]; incidence is broadly similar across the European Union when considered as a percentage of the population. In 2008, over 178,000 women will be diagnosed with invasive breast cancer in the U.S., almost 70% of which will be estrogen receptor-α positive (ER+; HUGO Gene Symbol = ESR1) [2,3]. The percentage of ER+ sporadic breast cancers increases linearly with age but even in premenopausal cases the proportion is high; 62% at age ≤35 and 72% by age 49 [2–4]. Data from randomized trials and meta-analyses clearly show that all breast cancer patients derive a statistically significant survival benefit from adjuvant chemotherapy, and that all hormone receptor positive breast cancer patients benefit from adjuvant endocrine therapy [5–9]. For postmenopausal women, the benefit from adjuvant Tamoxifen (TAM) is comparable to that seen for cytotoxic chemotherapy. While 5 years of adjuvant TAM produces a 26% proportional reduction in mortality [8], many ER+ tumors eventually recur [10]. Since advanced ER+ breast cancer largely remains an incurable disease, resistance to endocrine therapies is a significant clinical problem.

Endocrine therapy is administered as an antiestrogen (AE) like Tamoxifen (TAM) or Fulvestrant (FAS; Faslodex; ICI 182,780), or as an aromatase inhibitor (AI) such as Letrozole or Exemestane. It is less toxic and potentially more effective therapy in the management of hormone-dependent breast cancers. Antiestrogens, and TAM in particular, have been the “gold standard” first line endocrine therapy for over 30 years [11], clinical experience with this drug likely exceeding over 15 million patient years [10]. TAM increases both disease free and overall survival from early stage breast cancer, and it also reduces the incidence of invasive and noninvasive breast cancer in high-risk women [8,9]. Raloxifene, another antiestrogen, is effective in reducing the rate of postmenopausal bone loss from osteoporosis as well as the rate of invasive breast cancer [12]. Newer antiestrogens such as FAS show significant activity relative to TAM and some AIs [13,14]. Third generation AIs are now widely accepted as viable alternatives to AEs for first line endocrine therapy in postmenopausal women with metastatic disease; overall response rates are generally greater for AIs [15]. Importantly, tamoxifen is the only single agent with demonstrated efficacy in both premenopausal and postmenopausal women with invasive breast cancer. Other AEs and all of the AIs require the complete cessation of ovarian function.

Of current interest is identification of the optimum choice and scheduling of AEs and AIs. Evidence clearly shows improvements in disease free survival for combined adjuvant therapy (an AI and an AE usually given sequentially) over single agent TAM [16–20]. However, the ability of AIs to induce a significant improvement in overall survival compared with 5 years of TAM alone is uncertain [15]. In terms of metastatic disease, recent data imply that response rates with an AI are either equivalent with or higher than with TAM [21,22]. Given the increasing number of endocrine treatment options, there is a clear need to optimize the selection and scheduling of agents for both early stage and advanced disease. Whichever way these controversies are eventually resolved, it is clear that both AIs and AEs will remain as key modalities in the management of ER+ breast cancers. Unfortunately, the inability of endocrine therapies to cure many women with ER+ disease will also remain.

1.1. Endocrine resistance: receptor phenotypes

Several resistance phenotypes are evident from both experimental models and clinical observations. The two primary receptor phenotypes are ER+ and ER−. These receptor-based phenotypes have been further stratified by addition of the estrogen-regulated receptor for progesterone (PGR; HUGO Gene Symbol = PGR). The degree of treatment benefit from endocrine therapy varies according to receptor phenotype. For example, approximately 75% of ER+/PGR+, 33% of ER+/PGR−, and 45% of ER−/PGR+ cases of metastatic breast cancer respond to TAM [10]. Endocrine responses in truly ER− tumors are probably relatively rare and of uncertain relevance, as they most likely reflect incorrect assessments of what may be very low ER and/or PGR expression values. Data from the Early Breast Cancer Trialists' Collaborative Group meta analyses show that TAM therapy generates a non-significant 6% reduction in the 10-year risk of recurrence. A non-significant increase in the risk of death from any cause in patients with ER- breast cancer also was reported [8,9]. The real value of PGR, which is the only modification to this clinical prediction scheme for directing endocrine therapy to become routine in over 30 years (the value of directing endocrine therapy based on HER2 is still controversial), is largely limited to ER- tumors. It is general practice in the United States to treat all ER+ and/or PR+ invasive breast tumors with endocrine therapy. However, it remains impossible to predict whether an individual patient will receive benefit from treatment and the magnitude or duration of any benefit. Better predictors of each individual patient's endocrine responsiveness are clearly needed.

1.2. Endocrine resistance: pharmacological phenotypes

Several pharmacological phenotypes have been identified in experimental models of either human breast cancer cells growing *in vitro* or of xenografts in immune-deficient rodents [10]. These phenotypes include (i) estrogen-independent (which appears equivalent to AI resistance but is not so for antiestrogen resistance [23] – some breast cancers can become resistant to an AE but still respond to an AI and *vice versa*); (ii) estrogen-inhibited (recently identified in MCF-7 models [24]); (iii) TAM-stimulated (identified first in MCF-7 xenografts [25,26]); TAM-unresponsive but FAS sensitive [27] (identified first in MCF-7 models and subsequently observed in clinical trials [13]); TAM and FAS crossresistant [28] (perhaps this is truly antiestrogen crossresistant and it is seen both clinically in patients and experimentally in MCF-7 models [13,29]). Other variations on these phenotypes likely occur but are beyond the scope of our discussion.

1.3. Clinical evidence for the prevalence of pharmacological resistance phenotypes

Obtaining direct clinical evidence for the prevalence of each of the pharmacological resistance phenotypes is challenging. We have previously noted the utility of applying clinical responses to TAM withdrawal in metastatic breast cancer as one means to define, at least in broad terms, the likely relevance of a series of pharmacological phenotypes [29]. This approach is somewhat limited, as the number of cases across all studies is modest (n=241). Furthermore, TAM withdrawal responses cannot readily distinguish between TAM-stimulation and estrogen-inhibition because each should predict for a clinical benefit. The latter would induce a benefit because most breast cancers contain significant concentrations of 17β-estradiol, independent of both menopausal and ER/PGR status [10], sufficient to produce the estrogen-inhibited phenotype [24]. Indeed, the superiority of AIs over TAM in inducing clinical response strongly implies that over 75% of ER+/PGR+, at least 50% of all ER+ breast cancers irrespective of PGR expression, and 45% or more of ER−/PGR+ breast tumors are driven by adequate access to estrogen.

In our prior assessment, almost 9% of patients received an overall clinical response to TAM withdrawal (partial responses + complete responses). When disease stabilizations were included we estimated that less than 20% of patients received clinical benefit [29], suggesting

that the sum of TAM-stimulated plus estrogen-inhibited clinical phenotypes may not account for the majority of resistant phenotypes in women. Of course, given the number of ER+ breast cancers arising every year, these phenotypes are relevant to a notable number of women. The major response to TAM withdrawal was clinically detectable disease progression - greater than 80% of cases - strongly implicating unresponsiveness as the primary clinical resistance mechanism to TAM. Whether these breast cancers are fully crossresistant to all endocrine therapies, or retain sensitivity to AIs, cannot be determined from this analysis.

Nomura *et al*. [30] took a different approach and assessed the responsiveness to estrogen and TAM in short-term primary cell cultures of n=153 ER+ breast cancer biopsies. This approach allowed the authors to separate the various pharmacological phenotypes; approximately 7% of ER+ primary cultures were stimulated by TAM and almost 3% were inhibited by physiological concentrations of estradiol – notably close to our estimate of 9% for the sum of these two clinical phenotypes.

It is important here to separate responses to physiological estrogens from those produced by pharmacological estrogen therapy. High dose estrogen therapy was used prior to the advent of TAM. As with all endocrine therapies, approximately 30% of all breast cancers (receptor status was not available when most of these studies were done) responded [31,32]. Side effects were unfavorable, probably explaining the switch to TAM that also induces responses in approximately 30% of all breast cancers (when receptor status is not considered). It is also likely that the mechanisms of action of pharmacological and physiological dose estrogens differ. Over 15 years ago, we were the first to show that pharmacological concentrations of both estradiol and TAM induce changes in the membrane fluidity of breast cancer cells and that this correlates with changes in cell growth [33]. It is unlikely that membrane fluidity changes are major contributors to the action, either prosurvival or prodeath, of physiological estrogen exposures but they likely do contribute to the prodeath effects of pharmacological exposures.

2. Cell Fate in the Context of Endocrine Responsiveness

Therapeutic strategies for breast cancer generally aim to alter the balance between cell death and cell survival such that cancer cells (but ideally not normal cells) die. However, endocrine therapies consistently also induce a notable growth arrest in sensitive tumors. The relative importance of growth arrest and cell death remains unclear. To explore this issue, we will first discuss the forms of cell death and then compare the potential for cell death and cell growth arrest to contribute to endocrine responsiveness.

Cell death pathways include signaling to apoptosis, autophagy, mitotic catastrophe, necrosis, and senescence. Late events in cell death are reasonably well defined at the molecular (such as PARP cleavage) and cellular levels (including DNA disintegration). However, knowledge of the regulatory signaling upstream of these events, and how this signaling is integrated and processed, is now known to be incomplete. Mitochondrial function and integrity, regulated in part by BCL2 family members, are central to several forms of cell death [34–36].

2.1. Apoptosis

Apoptosis is a programmed cell death defined by morphological criteria related to organized chromatin condensation and fragmentation of the cell nucleus, accompanied by cleavage of DNA, formation of apoptotic bodies, cell shrinkage, and ruffling of the cell membrane [35, 37,38]. Two major pathways are involved. The *intrinsic (mitochondrial) pathway* is regulated by the proapoptotic and antiapoptotic BCL2 family members; this pathway involves changes in mitochondrial membrane permeability (MMP), release of cytochrome c, exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and the eventual loss of plasma

membrane integrity [39]. The *extrinsic (cell surface receptor) pathway* is dependent upon extracellular signals including tissue necrosis factor-α (TNFα), Fas ligand, and TNF-related ligand TRAIL [37,38]. The intrinsic and extrinsic pathways activate caspases, the "executioners" of apoptosis, which cleave DNA and catabolize the cytoskeleton. Apoptosis is not a discrete process and occurs over time - early (4–18 hrs), middle (18–36 hrs), and late stages (≥36 hrs) are often described based largely on data from cell culture models. Changes in specific BCL2 family members (early events that can precede changes in MMP), changes in MMP, and the exposure of phosphatidylserine are generally interpreted as representing early-to-middle apoptosis. Cytoplasmic cytochrome c release from mitochondria, changes in propidium iodide staining, increased terminal transferase dUTP nick end labeling (TUNEL) and cleavage of the DNA repair enzyme PARP-1 are associated with late apoptosis or necrosis [35].

2.2 Autophagy

Autophagy is a lysosomal pathway where cytoplasmic contents are degraded by double/multi-membrane vacuoles or autophagosomes, normally resulting in the removal of defective or damaged organelles, *e.g.*, mitochondria. A better understanding of the regulation of autophagy has recently begun to emerge; key regulators are now known to include BCL2 family members [40,41] and their interacting proteins such as Beclin-1/ATG6 (BECN1) [42]. BCL2 antiapoptotic proteins can block autophagy by inhibiting BECN1 [36]. Since monoallelic loss of the BECN1 locus is seen in >40% of breast cancers [43] (and in MCF-7 cells), modulating BCL2 may be an effective mechanism for regulating BECN1-activated autophagy. Autophagy can be identified by the absence of marginated nuclear chromatin, the presence of cytoplasmic vacuoles using transmission electron microscopy or monodansylcadaverine [44,45], cleavage of the LC3B protein [46,47], and regulation of the p62/SQSTM1 protein [48]. Early events in autophagy may be reversible; later events may (or appear to) share mechanisms with other cell death pathways. For example, cleavage of ATG5 by caplain [49] or upregulation of BID [41] can cause a switch from autophagy to apoptosis.

Paradoxically, autophagy can act as a cell survival mechanism when extracellular nutrients or growth factors are limited, or as an alternative cell death pathway to apoptosis [50]. Prosurvival outcomes likely reflect an adequate adjustment to stress, with energy/nutrients recovered from the organelles "digested" in the autophagosomes. Prodeath outcomes may arise when the self-digestion of autophagy leads to such a loss of organelles that the cell can no longer survive. In cancer cells, autophagy induction can accelerate cell death [51–55] or promote cell survival [56–58], independently or in response to treatment with cytotoxic agents.

2.3. Mitotic Catastrophe

Faulty DNA structure checkpoints, or the spindle assembly checkpoint, are key components of this form of cell death [59,60]. Disruption of the normal segregation of many chromosomes results in rapid cell death [59]. When this cell death does not occur, the cell can divide asymmetrically and produce aneuploid daughter cells [61] that can become neoplastic [59, 61]. Thus, mitotic catastrophe is characterized by multinucleation.

2.4. Necrosis

Necrosis is a chaotic process marked by cellular edema, vacuolization of the cytoplasm, breakdown of the plasma membrane, and an associated inflammatory response caused by the release of cell contents into the surroundings. Increased permeability to trypan blue or other vital dyes, in the absence of organized chromatin condensation and DNA fragmentation, is characteristic of necrosis [44,62].

2.5. Senescence

Senescent cells are characteristically enlarged, flattened with vacuoles and a large nucleus, be come permanently cell cycle arrested and unresponsive to mitogenic stimuli and express β-galactosidase [45,63]. Normally, as telomerase activity falls over time, successive telomere shortening limits proliferation and leads to “cellular senescence” or “mortality stage 1 (M1)”. Inactivation of p53 can by bypass M1 growth arrest, producing critically short telomeres and massive cell death called “mortality stage 2 (M2)” or “crisis” [64].

2.6. Endocrine-Induced Cell Death in Breast Cancer

Precisely how breast cancer cells die following estrogen withdrawal (or AI treatment) or AE treatment is unclear. Senescence may not be the dominant mechanism, since this process frequently involves DNA damage and p53 activation [38,45] but breast cancer cells respond to AEs and to estrogen withdrawal even if they have mutated p53 [35,65]. While apoptosis is clearly implicated [65–68], some of the apoptosis endpoints in prior studies may not distinguish among earlier events more closely implicated with signaling initiated through autophagy. Autophagy has been implicated in response to endocrine therapy [69–71] and we also see the induction of significant autophagy associated with endocrine therapies.

Fig 1 shows our ability to detect significant changes in the number of autophagosomes as measured by an increase in the presence of cytoplasmic vacuoles identified by monodansylcadaverine staining [44,45] (Fig 1), increased cleavage of the LC3 protein [46, 47], and reduced expression of p62/SQSTM1 [48,72–74] (Fig 2). We have previously shown, as have others, that AE treatment and estrogen withdrawal are also accompanied by increases in the level of apoptosis and growth arrest in sensitive cells. Indeed, when restoring AE sensitivity in resistant cells we frequently see that sensitivity is reflected in the restoration of an ability of the antiestrogen (or estrogen withdrawal) to both increase apoptosis and reduce proliferation [75,76]. As shown in Figs 1 and 2, and consistent with other reports [69–71], prodeath autophagy also is associated with the growth inhibitory effects of endocrine therapies in breast cancer cells. Thus in experimental models, cells responding to endocrine therapies concurrently experience an increase in cell growth arrest accompanied by both apoptosis and a prodeath autophagy.

2.7. Proliferation, Cell Death, and Endocrine Responsiveness

One of the most consistent observations in both experimental models *in vitro* and *in vivo* and in clinical specimens is the ability of endocrine therapies to induce a profound growth arrest in sensitive breast cancer cells. However, the relative importance of increased cell death compared with reduced proliferation is not entirely clear. In most endocrine sensitive experimental models, growth arrest and cell death concurrently occur and both clearly contribute to the ability of endocrine therapies to affect changes in anchorage-dependent cell number, anchorage-independent colony formation, or tumorigenesis over time [27,77,78]. Less clear is their relative contribution in driving clinical responses to endocrine therapies. Growth arrest appears to be readily detected in breast tumors responding to endocrine therapy. Less clear is the ability to detect robust changes in apoptosis. Some investigators do [79], and some do not [66], see an association of apoptosis or a molecular maker(s) of apoptosis with clinical response. The latter is in marked contrast to studies in experimental models. For some studies, response is related to molecular markers of apoptosis such as BCL2 [79] or the FasL:Fas ratio [80]. Notably, expression of the anti-apoptotic molecule BCL2 is reduced in responsive breast tumors by 3 months of TAM treatment [79], while in breast tumors that remain after TAM therapy BCL2 expression is elevated [81]. However, as noted above, BCL2 can affect both an apoptotic and autophagic cell death and its measurement alone is likely a poor predictor of any specific cell death mechanism.

If cell death does not occur in clinical breast cancer this observation clearly requires explanation. Several possible explanations exist – in the absence of compelling experimental/clinical data supporting or eliminating these explanations we make no assessment at this time on their relative merits. Firstly, it should be noted that measures of apoptosis are usually the primary endpoints for assessing rates of cell death. Our previously published results, the data in Fig 1 and Fig 2, and the work of others [69–71] show that estrogen withdrawal or antiestrogens increase both the rates of apoptosis and autophagy in breast cancer models responding to treatment. We interpret this as a prodeath autophagy in sensitive cells, consistent with other reports [69–71]. It remains unclear whether autophagy or apoptosis dominates as the cell death mechanism or whether this varies among different breast cancer cells. Measuring apoptosis may be the wrong measure of cell death in tumors, or it may be an inadequate measure if it represents only some proportion of cells that die through this process. Secondly, apoptosis is often considered to comprise early, mid and late stages, and an irreversible commitment to cell death may not be robustly associated with endpoints other than those definitively reflecting late stage apoptosis. A measure of apoptosis that is not robustly associated with ultimate cell death could provide an incomplete assessment of the rate or extent of cell death. Thirdly, if the timing of apoptosis is as fast in patient tumors as it is *in vitro*, measurements taken before 24–36 hrs and/or after 36–48 hrs could miss many of the key events. The most sensitive cells would have been through apoptosis and be already dead and gone, and the rate of apoptosis could have returned to the basal level. Fourthly, duration of the apoptotic response may differ between basal apoptosis and drug-induced apoptosis. If drug-induced apoptosis leads to a more rapid death, the number of cells processing though apoptosis could increase without any detectable change across time in the apparent rate of apoptosis.

Finally, a reduction in cell proliferation alone could be sufficient to account for some shrinkage of tumor size, as the rate of cell replacement might no longer be sufficient to account for cell loss from either a basal rate of cell death and/or loss to migration and metastasis. However, unless almost all growth arrested cells also undergo some form of cell death, it is unclear why growth arrest alone should lead to large and relatively rapid reductions in tumor size (over several weeks compared with often many years of presumably much longer growth prior to clinical detection and treatment). Growth arrest alone may be sufficient to account for good responses in some tumors, particularly where there is a high basal rate of cell death. However, it is not immediately clear how this applies to tumors with an inherently low rate of proliferation, whether because the growth fraction is large but cycling slowly or the growth fraction is small but proliferating rapidly. This is an area where mathematical modeling could be particularly useful, since it could compare the effect sizes needed for relative changes in proliferation and cell death to affect predicted overall tumor size over time.

While there is currently no definitive understanding of the primary cell death mechanisms in either experimental models or in breast tumors in women, or of the relative importance of endocrine therapy-induced changes in proliferation compared with cell death, there are potentially important implications for the underlying biology of the cancer cells. If the primary driver of response as seen in tumor shrinkage is a reduction in proliferation, this will leave many cells alive and still metabolically active. Surviving cells have the ability to adapt to the endocrine-induced stress and eventually overcome the proliferative blockade and grow – they will become resistant. This process seems unlikely to occur in many of those women who receive the clear long term benefit of a significant reduction in the risk of death [8,9].

Whether it is the growth arrested but surviving cells that eventually become resistant is unknown but it is certainly an intuitively satisfying hypothesis. Moreover, this hypothesis is supported by the ability to take sensitive cells in culture, expose them for prolonged periods to either estrogen withdrawal or AE treatment, and eventually induce an acquired resistant phenotype [27,28,77,82]. This process is accompanied by a profound and prolonged period of

growth arrest prior to the emergence of resistant cells, a pattern consistent with the clinical progress of the disease in tumors that initially respond to therapy but that eventually recur – often a decade or more after the initiation of TAM treatment.

3. Molecular Signaling and Resistance

The precise mechanisms of resistance to an AE and/or an AI remain unclear, reflecting an incomplete understanding of the signaling affecting cell proliferation, survival, and death and their hormonal regulation in breast cancer. We have previously reviewed the mechanisms of resistance to AEs and to estrogen deprivation elsewhere in some detail [10,23,29], so we focus here on the molecular signaling aspects of resistance and how these may be integrated and explored using emerging technologies. We will focus primarily on signaling to cell death – signaling to regulate proliferation in the context of endocrine responsiveness will be the subject of a separate review.

The primary technologies that have matured sufficiently to enable global approaches to network modeling include gene expression microarrays, ChIP-on-chip, SNP chips, high throughput DNA sequencing, and array CGH. Each of these technologies has reached a high level of maturity, and each is characterized by the generation of very high dimensional data on each sample whether the read-out be genomic or transcriptomic data; this also is true of the emerging high-throughput proteomic technologies. The remarkable volume of data, and the diversity of biological information that informs the interpretation of these data, has begun to transform the fields of biostatistics, computer science, and bioinformatics. However, the properties of these datasets are often not fully understood nor are the challenges these properties provide for data analysis and network modeling. Readers interested in exploring some of these challenges can read recent reviews [83,84]. Here we will address briefly several approaches to the use of these data for network modeling.

3.1. A network signaling hypothesis of endocrine responsiveness

Estrogen-independence and AE resistance are complex phenotypes and both genomic and non-genomic activities are implicated [10,33,85]. We consider it unlikely that endocrine resistance in ER+ tumors is driven by a single gene/signaling pathway. Unlike many previous single gene/pathway studies, our central hypothesis invokes a gene network that confers diversity and redundancy in signaling [10,86]. The cell death/survival network incorporates specific signaling as affected by estrogen and AE modification of ERα function. Thus, AEs regulate this network differently than other agents such as cytotoxic drugs.

Signaling leads first to the reversible initiation of several cell death/survival signaling pathways within the network. The irreversible machinery of cell destruction is activated at some later point. This machinery may induce common outcomes - such as activation of effector caspases and DNA/plasma membrane disintegration - independent of the early specific initiating signals. Hence, we envision multiple concurrent signals processing through this network, some prosurvival and some prodeath, with cell fate reflecting the dominant signaling. In endocrine resistant cells, endocrine regulation and/or function of components of this network are changed and prodeath signals are either no longer induced or dominant.

This cell fate signaling network hypothesis is intuitively logical and certainly testable. Evidence that cells induce prosurvival signaling in an attempt to circumvent stressors implies that some cells are successful and ultimately survive whereas others are unsuccessful and die. Thus, the balance between prosurvival and prodeath signaling is likely the final arbiter of cells fate [83]. While this remains an area of active investigation, we first discuss the basic principles of network modeling and then provide an example of a seed-gene network of endocrine regulated signaling in endocrine responsiveness.

3.2. Basic Concepts of Gene Networks

Cellular signaling occurs more in the context of interactive networks than through linear pathways [83]. The basic topology of a network is defined by nodes (genes/proteins) and their interconnections (edges). Interconnections are multi-faceted and include one-to-one, one-to-many, or many-to-one relationships, and feed-forward or feed-back loops. The dynamic activity of a network is constrained by the various forms of interactions, and the network behaves only in certain ways and controlled manners in response to changing cellular conditions or external stimuli [87]. While often built solely from gene expression microarray data, these data are high dimensional and contain spurious correlations that can confound simple solutions for network building [83,84]. Relevant events also occur in the genome and proteome, some of which can affect the transcriptome. For example, a transcription factor (TF) may be activated by phosphorylation and bind to responsive elements in the genome but the regulation of its downstream targets is seen in the transcriptome [83]. An example of this relationship is the ligand independent activation of ERα following its phosphorylation on SER118 by MAPK [88].

Simplistically, there are two basic approaches to network modeling of high dimensional data: top-down and bottom-up. The former is probably the most widely used approach as several accessible commercial software packages are available that make this an easy task to perform without the need for training in biostatistics or bioinformatics. These packages often apply various implementations of gene ontologic and semantic search algorithms that identify cellular functions and pathways to which individual nodes are assigned; these data are then graphically represented.

The solutions produced by several popular top-down algorithms are often characterized by representations of tens-to-hundreds of nodes linked by hundreds-to-thousands of edges, making interpretation challenging (Fig 3). Whether the algorithms address the confounding properties of high dimensional spaces, such as the curse of dimensionality or the confound of multimodality, or incorporate the critical aspects of cellular context and alleviate the trap of self fulfilling prophesy, is not clear [83]. Amongst the additional challenges are the incompleteness of relevant biological knowledge and the annotation error rate in the source databases searched by these algorithms [83]. Nonetheless, these approaches can be useful when carefully applied and their limitations fully understood, and when experts from both the biological and mathematics domains combine expertise to assess the validity of the solutions. Currently, such approaches probably have most to offer in the area of hypothesis generation, rather than in the construction of truly biologically meaningful signal transduction networks.

3.3 The “Seed-Gene” Approach to Network Modeling

The bottom-up approach is generally referred to as the “seed-gene approach” to network modeling [89]. This approach requires the extraction of a small number of seed-genes from within the primary data; these genes are then used to grow the network in several ways. We will not address all the various approaches in this review but provide a few brief examples. Various modeling methods can be applied to find and link adjacent nodes, growing the network *de novo*. Local subnetworks can be identified and overlaid or linked to the initial seed genes. A simple approach is the incorporation of a canonical pathway (which may be a subnetwork in what would be a final and much broader network) when it is known to be relevant in the cellular context under study and where incorporating the nodes and edges of the canonical pathway members is consistent with statistical properties of the growing model topology.

Knowledge of how a gene (node) affects the expression/function of another node provides directional connectivity information that can be applied to the interacting nodes. Transcription networks can be grown (or transcriptional edges between nodes in a network that incorporates

other biological knowledge) by linking TFs to their downstream targets. These targets can be predicted using specific algorithms [90–93]; where possible it is preferable to incorporate functional data such as that obtained from ChIp-on-chip arrays [91]. Thus, interacting nodes can be identified along with the directionality of their edges as the seed gene network is grown.

The most labor intensive approach is to derive experimentally nodes and edges, growing the network using definitive laboratory-derived knowledge. Where additional high throughput data are already available, such as ChIp-on-chip, this is preferable. Currently, functional data is probably more often obtained one gene at a time, using standard molecular methods such as gene knock-down and over-expression. This laborious approach is becoming supplanted with the emerging functional genomic methods such as siRNA, ribozyme, or antisense libraries that can test experimentally the contribution of hundreds to thousands of genes. These methods enable investigators to extract concurrently nodes that experimentally generate biologically appropriate changes in the phenotype under investigation.

Once seeds and their edges are identified, and functional biological metadata obtained, interactive models can be grown using neural network and other machine learning tools. Several models have been proposed to reveal the behaviors of regulatory networks from gene expression data [22,23] including Boolean networks [24–26], Bayesian networks [27–30], linear additive regulation models [31,32], state-space models (SSMs) [33,34], and recurrent neural networks (RNN) [35,36]. However, these methods use only mRNA expression data to infer networks.

Integrated approaches have been recently proposed to learn transcriptional regulation from various data sources [27,30,37–43]. An iterative search on mRNA expression and ChIP-on-chip data [37], or the incorporation of expression profiles, ChIP-on-chip, and motif data [41] have each been used in yeast to discover transcriptional networks. Several linear models or matrix decomposition methods have also been proposed [43–46]. Network component analysis (NCA) is a notably powerful approach [45] but NCA and these other methods cannot easily infer regulatory networks in biological systems more complex than yeast.

Other limitations exist in network modeling. Complete biological knowledge for topology estimation (node-node edges and directionality), such as high-throughput ChIP-on-chip data or functional data from laboratory experiments, are often not (or only partially) available for human cells. When heterogeneous data sources are integrated for computational inference, the consistency of different data sources is often inadequate or unknown. Topological knowledge also comes from biological experiments, which often contains false positives/negatives that can lead to incorrect network inference.

4. Seed-gene model for cell signaling and the regulation of cell fate

While we continue to develop new methods for network modeling, we have yet to report our modeling approaches to our own expanding data sets. Hence, we will here describe our initial studies on the use of seed-genes and experimental data to construct a simple wiring-diagram of our initial seed-gene network. The inability to induce signaling to irreversible cell death is a central component of drug resistance [94]. Thus, we propose that cells possess a common cell death/survival regulatory decision network of integrated and/or interacting pathways (see above).

Prior to building network models, it is necessary to extract initial nodes (seed genes) from which a network can be built [95]. Since ER is a TF and regulates other functionally relevant TFs that influence endocrine responsiveness and cell fate, selecting a small number of TFs as seed genes is reasonable for network modeling. The full list of *relevant* ER-regulated TFs that may affect cell fate is unknown. Nonetheless, our published data support the central hypothesis

that that IRF1 [65,96–98], XBP1 [76,96] and NFκB (RELA) [75,96] are key regulatory nodes or control key modules in this network. Moreover, our experimental data in endocrine sensitive and resistant breast human cancer cells now allow us to map their edges and directionality, in an appropriate cellular context, with some confidence.

4.1. X-Box Binding Protein 1 (XBP1) and the Unfolded Protein Response (UPR)

UPR is a central component of the endoplasmic stress response [99]), an adaptive signaling pathway that allows cells to survive the accumulation of unfolded proteins in the endoplasmic reticulum lumen [100]. Initially a compensatory mechanism allowing cells to recover normal endoplasmic reticulum function, a prolonged UPR may induce cell death. UPR, which can be induced by cellular stressors such as hypoxia, is activated by each of three molecular sensors: IRE1α, ATF6, PERK [101]. XBP1's *unconventional* splicing (occurs in the cytosol) by IRE1α is an obligate component in both IRE1α- and ATF6-induced UPR [101,102]. The UPR (initiated by XBP1 splicing by IRE1α) can activate autophagy [103]. Whether this is a prosurvival or prodeath form of autophagy is unknown, since UPR activation also can induce both prodeath and prosurvival outcomes [104].

XBP1 is a transcription factor that belongs to the basic region/leucine zipper (bZIP) family [105,106]. The unspliced form, XBP1(U), has a molecular weight of ~33 kDa and acts as a dominant negative of spliced XBP1 [107,108]. The spliced form, XBP1(S), has a molecular weight of ~54 kDa; splicing removes a 26 bp intron and creates a translational frame-shift. Regulation of transcription by XBP1(S) is a consequence of its homodimers activating specific cAMP response elements (CREs) with a conserved ACGT core sequence GATG**ACGT**G(T/G) NNN(A/T)T - sometimes called the UPR element [104,105,109]. XBP1(S), which is implicated in affecting plasma cell differentiation [110], is essential for fetal survival, neurological development, bone growth, immune system activation, and liver development [111,112]. XBP1 is also rapidly induced in response to estrogen-stimulation [113,114]. Consistent with the work of others [109], we have shown that XBP1(S) can bind to and activate ERα in a ligand-independent manner (Fig 4).

We have recently shown that XBP1(S) confers E2-independence (effectively an AI resistant phenotype) and AE crossresistance (TAM and FAS crossresistance) in both MCF-7 and T47D human breast cancer cells [76]. This activity appears to be driven primarily by XBP1(S), as introduction of the full-length XBP1 cDNA in either MCF-7 or T47D cells generates predominately the XBP1(S) protein. This observation suggests that the basal activity of IRE1α is already adequate and that XBP1(S) is the rate limiting protein. XBP1 is the only known substrate for the IRE1α endonuclease and only IRE1α can splice mammalian XBP1. Since XBP1 splicing is thought to function primarily within the UPR, breast cancer cells may be primed to respond to multiple stressors by activating a prosurvival induction of UPR.

4.2. Interferon Regulatory Factor-1 (IRF1)

RFLP linkage analysis assigned the IRF1 gene to 5q23-31; more definitive studies identified the locus as 5q31.1 [115]. IRF1 was initially identified because of its transcriptional activation of type I interferon (IFN) genes. We first showed the ability of interferons to sensitize breast cancer cells to TAM over 20 years ago [116]. More recently, IRF1 was implicated in T-cell development [117], and it is now known also to coordinate expression of the immunoproteasome [118], to regulate human telomerase activity [119,120], and to regulate key aspects of DNA damage repair [121,122]. Loss of IRF1 increases tumorigenicity in mouse models driven by ras or loss of p53 [123]. These activities may reflect IRF1's ability to signal to apoptosis [124], which can occur in a p53-dependent or -independent manner [121,125], with or without induction of $p21^{cip1}$ [125] or $p27^{kip1}$ [126], and through caspase-1 [121], caspase-3 [97], caspase-7 [97,127], caspase-8 [97,128], and/or FasL [129].

Following our initial observations of IRF1's likely role in breast cancer [130–132] and antiestrogen resistance [130], we confirmed its functional involvement using a dominant negative approach (dnIRF1) [65]. IRF1 and dnIRF1 induce opposing effects on proliferation *in vitro* and tumorigenesis *in vivo* through regulation of caspases-3/7 and caspase-8 activities [97]. These observations are consistent with the effects of inoculating an adenoviral vector containing IRF1 directly into mouse mammary tumors [133]. While p53-dependent apoptosis occurs in the breast [134], T47D cells express mutant p53 and our data show that intact p53 is not required for the proapoptotic actions of IRF1 [65,97]. In AE sensitive breast cancer cells, inhibition of AE-induced IRF1 activity by dnIRF1 is accompanied by reduced proapoptotic activity [65]. These observations on IRF1 and AE responsiveness have been confirmed and extended by others in both normal [135] and other neoplastic breast cell culture models [136, 137]. IRF1, which can signal through both p53-dependent and p53-independent mechanisms [121,125], provides a new and potentially important signaling molecule for integrating and regulating breast cancer cell survival in response to AEs

4.3. Nuclear Factor kappa B (NFκB)

The NFκB p50/p65 heterodimer complex comprises two homologous proteins; the p50 product of its p105 precursor (NFκB1; chromosome 4q24) and the p65 (RELA; 11q13). NFκB is maintained in the cytosol in an inactive state, bound with members of the IκB family that inhibit nuclear transport or block NFκB's nuclear translocation signal [138]. Activation usually proceeds by the IKK kinase complex phosphorylating IκB, resulting in IκB ubiquitination and degradation [139]. NFκB (RELA/NFκB1) is implicated in several critical cellular functions [140]. Reflecting its regulation by both estrogen and growth factors [141,142] that are involved in endocrine resistance [10,143], normal mammary gland development is dependent upon NFκB [144]. Increased NFκB activity arises during neoplastic transformation in the rat [145] and mouse mammary gland [146]. Upregulation of NFκB is associated with E2-independence [141,144]. The predominant NFκB form in breast cancer cell lines is RELA/NFκB1; the p52 family member also is expressed in some breast cancers [147].

We have shown that NFκB can confer estrogen-independence and AE crossresistance [75, 96,148]. Estrogen independent growth *in vitro* and *in vivo* is supported by increases in both NFκB DNA binding activity and expression of BCL3 [148]. This study highlights the functional implications of NFκB in AI resistance. Expression of IκBα (NFκB repressor) in estrogen independent LCC1 cells (LCC1 cells are derived from MCF-7 and are estrogen-independent but sensitive to AEs [149]), which have increased NFκB activation relative to estrogen-dependent MCF-7 cells, eliminates their estrogen-independence *in vivo*.

LCC9 cells (TAM and FAS crossresistant variant of LCC1 [28]) exhibit a further increase in NFκB expression and activation relative to LCC1 cells, apparently driven by increased expression of NEMO [75]. These observations imply that the level of activity in LCC1 cells is adequate for estrogen-independence but not AE resistance. Increased activation of NFκB [96] and loss of its antiestrogenic regulation in LCC9 cells [75] suggest that these cells might be dependent upon NFκB for survival/growth. Thus, we compared the growth response of LCC1 and LCC9 cells to vehicle or parthenolide (300 nM, 600 nM), a small molecule inhibitor of NFκB [150]. Parthenolide produces a dose dependent inhibition of MCF7/LCC9 cells with an apparent IC_{50} of approximately 600 nM ($p<0.01$ at both 300 nM and 600 nM parthenolide). In marked contrast, parthenolide does not affect growth of LCC1 cells at either of these concentrations [75]. We next asked if parthenolide can re-sensitize LCC9 cells to FAS-mediated apoptosis. FAS and parthenolide synergize to induce LCC9 cell death [75]. Since FAS alone is inactive [28], this synergism reflects at least a partial reversal of the FAS resistance component of the LCC9 cell phenotype and implicates NFκB as a key determinant [75]. Thus,

AE crossresistant cells exhibit a greater reliance upon NFκB signaling for proliferation, and inhibition of NFκB restores their sensitivity to apoptosis induced by FAS [96].

4.4. Expression of ER, PGR, XBP1, NFκB and IRF1 in breast tumors

Using gene expression microarrays, we previously compared the global structures of the transcriptomes of three ER+ human breast cancer cell lines (MCF-7, T47D, ZR75-1) and 13 human breast tumors (11 ER+; 2 ER−) and showed these to be notably similar to ER+ breast tumors from patients [151]. The striking similarities between cell lines and tumors are supported by a report that the estrogen-regulated genes in these cell lines are similarly regulated in breast tumors [152]. These data show that ER+ breast cancer cell lines and ER+ breast tumors in women share global similarities in the structures of their respective transcriptomes [151], and that these cell lines are appropriate models in which to identify clinically relevant endocrine-regulated molecular events [151,152]. Nonetheless, it is necessary to show that the seed genes we have selected are likely to be relevant to the biology of ER+ breast tumors.

To begin to explore the possible clinical relevance of these functional studies, we first asked if we could detect XBP1, NFκB, and IRF1 in breast tumors. We then asked whether any of these proteins were coexpressed in patterns consistent with the experimental data from cell lines. Using a series of breast cancer tissue arrays comprising 480 cores from 54 breast carcinomas (mostly ER+ tumors), we applied immunohistochemistry to explore the expression of the seed genes [153]. Pairwise correlation analyses cannot account for the possibility that unknown associations among proteins may confound each other, so we applied a novel use of partial correlation coefficient analysis. Partial correlation analysis allows an estimate of the correlation between two variables while controlling for a third, fourth and/or fifth and is particularly useful in the analysis of small signaling networks of 3–5 variables [154].

We confirmed the well established co-expression of ERα and PgR, implying that the samples are representative of most ER+ breast cancers. XBP1, NFκB, and IRF1 are each found in a high proportion of breast tumors [153]. Total XBP1 was measured, as XBP1(S) antibodies were not then available. XBP1 staining is variable but detectable in 79% of breast tumors. A very recent study has reported a significant association between XBP1(S) mRNA and poor response to endocrine therapy [155] – entirely consistent with our studies in breast cancer cell lines [76]. 57% of the tumors express detectable RELA in their neoplastic cells, similar to a prior study of n=17 breast tumors [147].

Expression of several of the proteins is correlated in breast tumors. IRF1 correlates with ER and PGR, and also with RELA and XBP1. While, these correlations depend on the subcellular localization of IRF1 and some are direct and others inverse correlations, they are fully consistent with the interpretation that these expression patterns reflect functionally relevant signaling links. For example, we might predict that IRF1 sequestered in the cytosol, unlike that in the nucleus, cannot act as a proapoptotic TF (the full coexpression patterns are described detail in the report by Zhu *et al.* [153]). We also find coexpression of XBP1 and RELA, consistent with the observation that XBP1 may be downstream of NFκB [110]. When each of the significant correlations is examined in the partial correlation coefficient models, the IRF1, NFκB, and XBP correlations remain [153]. These data are consistent with these three reflecting some component of a larger signaling network active in some ER+ breast cancers and further support their selection as seed genes from which to grow this network and understand its topology and function. Moreover, the functional data from our experimental models implies that this network links signaling and function through two key subcellular components – mitochondria and the endoplasmic reticulum.

4.5. Simple representation of a seed gene network of XBP1, NFκB and IRF1 based on functional data obtained from an appropriate cellular context

The experimental data supporting the wiring diagram representation of the network model shown in Fig 5 are discussed the preceding sections. Here we discuss how the signals may flow through this network. The three primary seed genes of IRF1, XBP1, and NFκB are evident as previously proposed [96]. IRF1 expression is repressed in resistant cells [96] but induced by antiestrogens in sensitive cells [65]. A dominant negative IRF1 confers an antiestrogen resistant phenotype, implying that IRF1-driven prodeath signaling is key to the regulation of cell fate [65].

In addition to changes in the expression of IRF1, the upregulation of NPM expression [96, 156] could also affect IRF1 action. Both NPM and IRF1 are estrogen-regulated genes in MCF-7 cells, IRF1 expression being suppressed, whereas NPM is induced [130,156]. Since NPM inhibits the transcription regulatory activities of IRF1 [157], the increase in NPM expression could bind remaining IRF1 and inhibit its ability to initiate an apoptotic caspase cascade. We also cannot exclude the possibility that NPM has activities independent of blocking IRF1, since NPM overexpression is sufficient to transform NIH 3T3 cells in a standard oncogenesis assay [157]. Increased levels of serum autoantibodies to NPM predict recurrence on TAM 6-months prior to clinical detection [158].

IRF1 and NFκB are known to form heterodimers and to regulate directly gene expression [159,160] including that of the inducible nitric oxide synthase promoter [159]. Since we do not know if it is primarily the gene regulatory effects of these heterodimers, or if their subcellular location is key (they act by preferentially sequestering one or the other so that transcriptional regulation does not occur), this is shown as a dotted line. We would predict, based on the inverse expression between NFκB and IRF1 in LCC9 cells [96] and in some breast cancers [153], that either the prodeath effects of any remaining IRF1 are being sequestered by NFκB in resistant cells and/or that the overexpression and activation of NFκB leads to a dominance of its prosurvival activities. The increased sensitivity of resistant cells to parthenolide is consistent with the functional relevance of at least the latter signaling outcome [75].

We have previously shown that the upregulation of NFκB in antiestrogen resistant cells [96] is likely driven in part by increased NEMO/IKKγ activity [75]. The prosurvival activities of NFκB are well documented [161]. Precisely how NFκB regulates cell survival remains to be fully established but activation of prosurvival members of the BCL2 gene family are involved in both acquired estrogen-independence [148] and antiestrogen resistance [75,76]. While NFκB is predicted to induce transcription of XBP1 [110], we have yet to report this direct regulation in breast cancer cells (studies are in progress). Whether or not this occurs, XBP1 is clearly upregulated in resistant cells [96] and this activity is sufficient to confer both estrogen-independence and antiestrogen resistance [76]. More recently, increased XBP1 mRNA expression has been show to predict for a poor response to TAM in breast cancer patients [155].

The central role of XBP1 within the UPR clearly implicates UPR activation in responsiveness to both estrogen-withdrawal and antiestrogen treatment [76]. UPR also is known to induce autophagy [103], although whether this is a prosurvival or prodeath autophagy remains unclear in the context of determining endocrine responsiveness. Autophagy is regulated, at least in part, by the action of BECN1. BECN1 activity is regulated by BCL2, which binds BECN1 and can block BECN1-mediated autophagy [36].

The regulation of BCL2 family members (BCL2, BCL3, and probably others) whether by IRF1, NFκB, and/or XBP1, can affect both autophagy and the intrinsic apoptosis pathway. The

intersection of their signaling at BCL2 family members, as shown in Fig 5, is one location within the broader network where the balance between prodeath and prosurvival signaling, and whether prodeath is autophagic or apoptotic, is determined. This intersection also links signaling through the UPR and endoplasmic reticulum to the mitochondria with the cell fate decision mechanisms - at least in the context of determining cell fate in the context of endocrine responsiveness in breast cancer. The signaling depicted in Fig 5 represents only a small component of this broader network. Nevertheless, this initial wiring diagram is consistent with a body of functional data in experimental models and it provides sufficient seed genes, their edges, and the directionality of these edges, to begin a more detailed exploration of this central network. Understanding this network's topology and function will lead to better candidates for drug discovery and to better algorithms to predict how individual tumors will respond to specific endocrine therapies.

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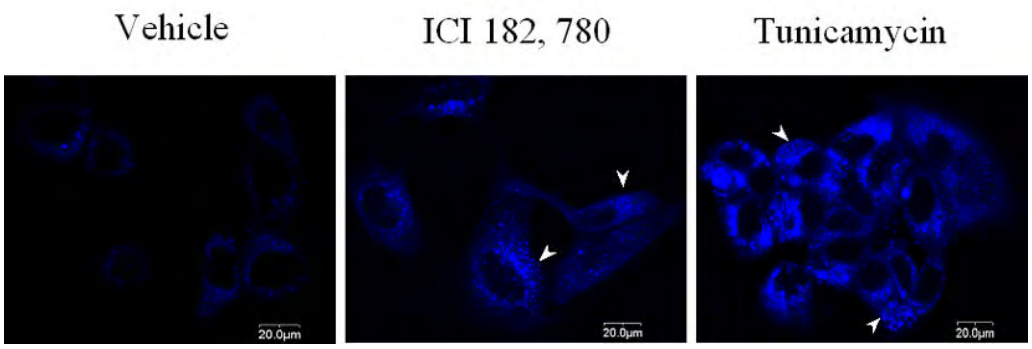

Fig 1.

Autophagy is enhanced upon FAS treatment in ER+ breast cancer cell lines. MCF-7 cells were treated with FAS (ICI 182,780), the endoplasmic reticulum stress and autophagy inducer tunicamycin (TUN), or ethanol control (vehicle) prior to staining with monodansylcadaverine (MDC). Increased MDC staining indicates that autophagy has been induced.

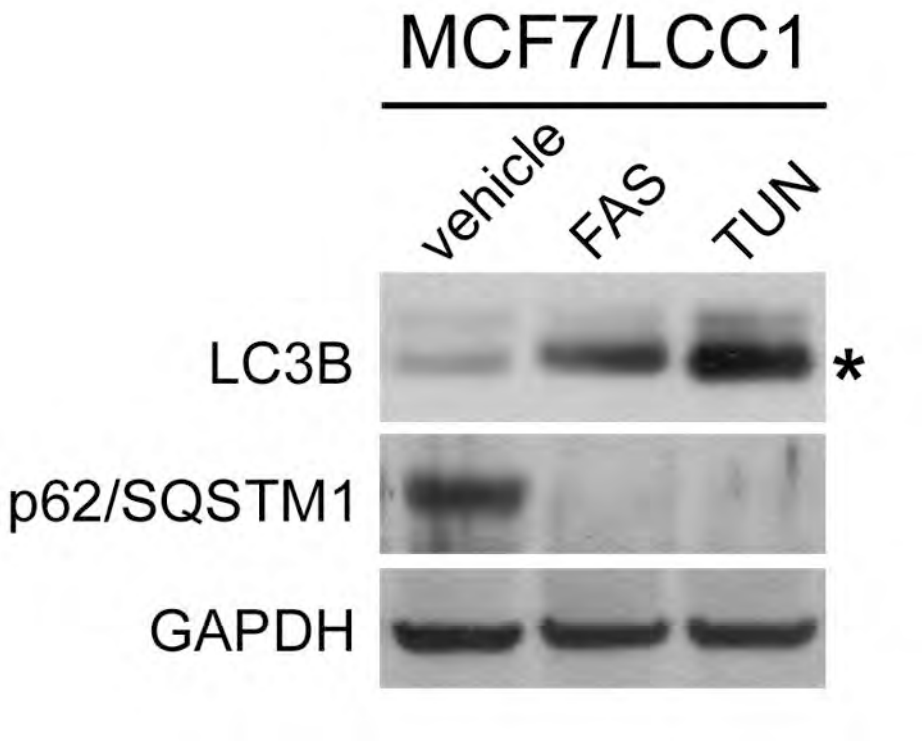

Fig 2.

Autophagy is enhanced upon FAS treatment in ER+ breast cancer cell lines. MCF7/LCC1 cells were treated with FAS, TUN, or vehicle prior to lysis and immunoblotting using standard procedures. Increased LC3BII (asterisk) and decreased p62/SQSTM1 expression both indicate that autophagy has been induced.

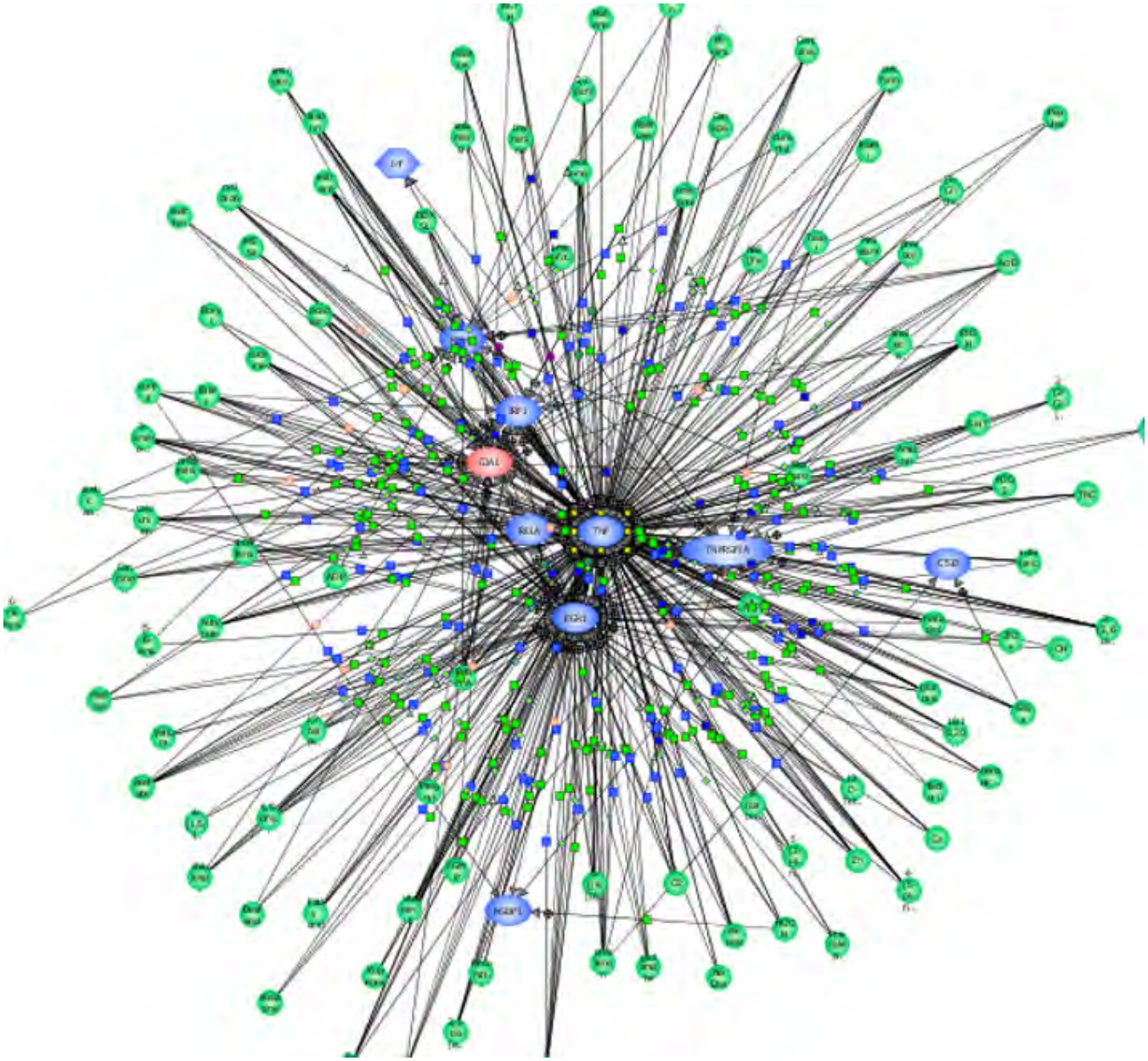

Fig 3.

Illustration of the complex and challenging nature of pathway analysis. Genes identified as being differentially expressed in resistant MCF7/LCC9 cells by SAGE and gene expression microarray were analyzed by Pathway Architect (Stratagene) to identify relationships *in silico*.

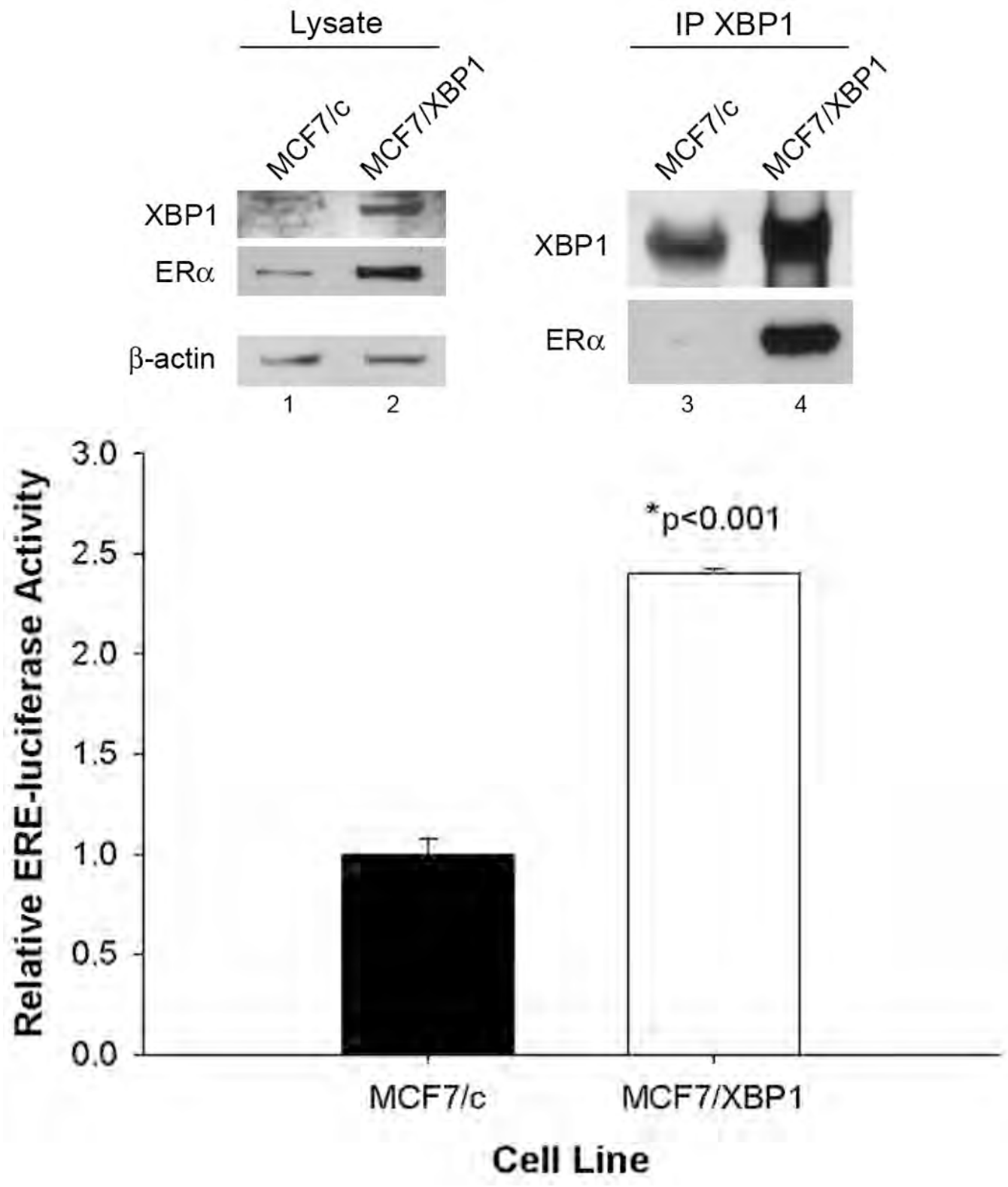

Fig 4.

Physical association of XBP1 and ERα is accompanied by robust ERE-driven transcriptional activity in MCF7/XBP1 cells. A, MCF-7 cells stably expressing XBP1 cDNA or the empty vector control (c) were treated with FAS or ethanol control (ctrl.) vehicle prior to lysis and immunoblotting (lanes 1 and 2) or co-immunoprecipitation of XBP1 and ERα (lanes 3 and 4) using standard procedures. B, MCF7/c and MCF7/XBP1 cells were transiently co-transfected with plasmids encoding 3xERE-luciferase and phRLSV40-Renilla for 24 hours prior to lysis and promoter-reporter luciferase assay by standard methods. Data are presented as mean relative ERE-luciferase activity ± SE for a representative experiment performed in triplicate, *$p<0.001$.

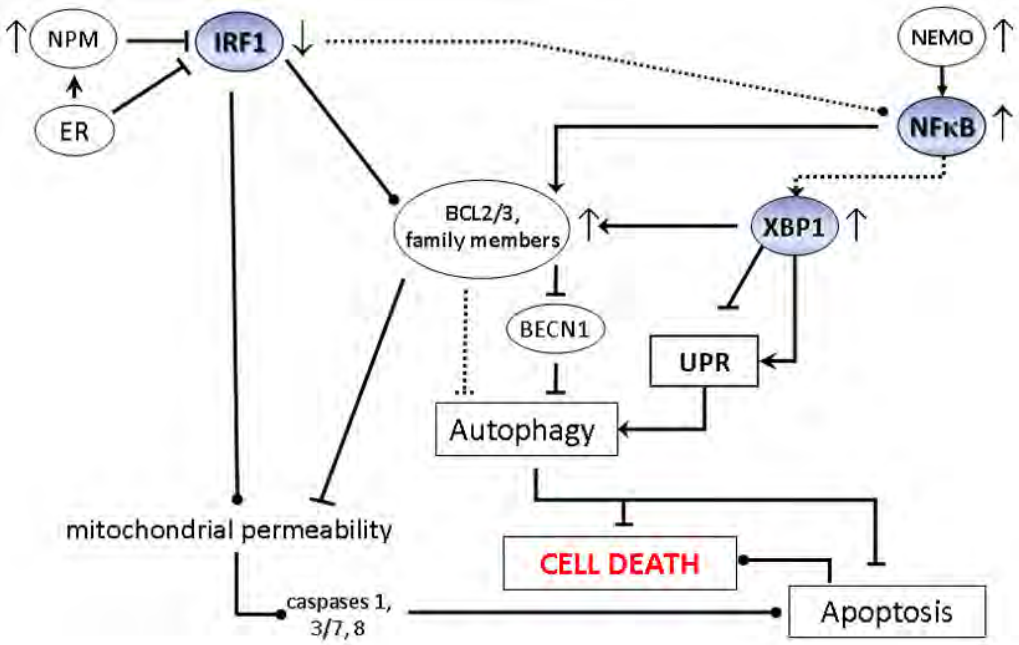

Fig 5.

Endocrine resistance seed gene network. Simple representation of a seed gene network of XBP1, NFκB and IRF1 based on functional data obtained from an appropriate cellular context (resistant MCF7/LCC9 cells).